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(21) International Application Number: PCT/US93/01207 (22) International Filing Date: 10 February 1993 (10.02.93) (30) Priority data: 07/833,429 10 February 1992 (10.02.92) US (71)(72) Applicant and Inventor: HAYNES, Barton, F. [US/US]; 4923 Wentworth Drive, Durham, NC 27707 (US). (74) Agents: WILSON, Mary, J. et al.; Cushman, Darby & Cushman, 1100 New York Avenue, N.W., Washington, DC 20005 (US). (81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).		Published <i>With international search report.</i>
(54) Title: USE OF SYNTHETIC PEPTIDES TO INDUCE TOLERANCE TO PATHOGENIC T AND B CELL EPITOPES OF AUTOANTIGENS (57) Abstract The present invention relates, in general, to the use of synthetic peptides to induce tolerance to immunogenic peptides. In particular, the present invention relates to a method of inducing tolerance in a mammal to an immunogenic peptide or protein comprising administering to a mammal a synthetic toleragen comprising a hydrophobic peptide linked to the N-terminus or C-terminus of the immunogenic peptide or protein, under conditions such that the tolerance is induced.		

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USE OF SYNTHETIC PEPTIDES TO INDUCE TOLERANCE TO PATHOGENIC T AND B CELL EPTOPES OF AUTOANTIGENS

BACKGROUND OF THE INVENTION

5 This is a continuation-in-part of
Application Number 07/833,429, filed February 10,
1992, which is a continuation-in-part of
Application No. 07/591,109, filed October 1, 1990,
which is a continuation-in-part of Application
10 Number 93,854, filed September 8, 1987, now U.S.
Patent 5,019,387, the entire contents of which are
hereby incorporated by reference.

Field of the Invention

15 The present invention relates, in
general, to the use of synthetic peptides to
induce tolerance to immunogenic peptides. In
particular, the present invention relates to a
method of inducing tolerance in a mammal to an
immunogenic peptide or protein comprising
20 administering to a mammal a synthetic toleragen
comprising a 2 to 20 amino acid hydrophobic
peptide linked to the N-terminus or C-terminus of
the immunogenic peptide or protein, under
conditions such that the tolerance is induced.

25 Background Information

Many autoimmune diseases in animals and
man are characterized by T and B cell responses to
pathogenic epitopes on self antigens
30 (Immunotherapy of Diabetes and Selected Autoimmune
Diseases, G.S. Eisenbarth (ED) CRC Press, Boca
Raton, 1989; Current Therapy in Allergy,

Immunology, and Rheumatology-3. L.M.

Lichtenstein, et al, B.C. Decker, Inc., Toronto, 1988). Examples of autoimmune diseases or disease models that are caused by autoreactive B cells responses are listed in Table 1. Examples of autoimmune diseases or disease models that are caused by autoreactive T cell responses are listed in Table 2. A method to tolerize human lymphocytes to not respond to pathogenic T and B cell epitopes of autoantigens that otherwise induce immune responses that cause tissue damage would represent a significant advance in the therapy of autoimmune diseases. Similarly, multiple clinical situations exist outside the setting of autoimmune disease, in which B and T cell responses are harmful and would advantageously be shut off or decreased. Examples of pathogenic non-autoimmune antibody responses are antibody responses to ABO incompatible erythrocytes. A method to induce tolerance against this type of immunogen would be a powerful tool for treatment of a number of similar conditions.

Recently, it has also become clear that tissue destruction in certain infectious diseases is caused by immune responses against normal tissue that are induced by infectious agents. For example, in HTLV-I infection, the clinical syndrome of HTLV-1 associated myelopathy (HAM) has been shown to be associated with the induction of cytotoxic T lymphocytes reactive with a specific region (SP4A1) OF HTLV-1 gp46 envelope glycoprotein (S. Jacobson, et al, J. Immunol. 146:1155-1162, 1991). Similarly, lymphocytic pneumonitis in HIV infection has been shown to be associated with the presence in lung lymphocytes of CTL specific for HIV infected cells (AIDS,

B.D. Walker, et al, 4:177, 1990). In both HIV and HTLV-1 infection, it is thought that certain manifestations of the disease are caused by the induction of anti-viral immune responses that cross-react with normal human host antigens (G.W. Hoffman, et al, Proc. Natl. Acad. Sci. USA 88:3060-3064, 1991; H. Wigzell, et al, FASEB J. p.2406-2410, 1991; H. Golding, et al, J. Clin. Invest. 83:1430-1435).

Robinson et al have demonstrated that antibody responses to HIV envelope gp41 epitopes enhance HIV infectivity (W.E. Robinson, et al, Proc. Natl. Acad. Sci. USA, 86:4710, 1989). Recently, evidence has been presented that many if not all of the manifestations of AIDS may be caused by an autoimmune response to HLA antigens that are induced by HIV viral proteins that share sequence homologies with normal host HLA molecules (G.W. Hoffman, et al, Proc. Natl. Acad. Sci. USA 88:3060-3064, 1991; H. Wigzell, et al, FASEB J. p.2406-2410, 1991; H. Golding, et al, J. Clin. Invest. 83:1430-1435). Thus, a method of induction of tolerance (non-responsiveness) to pathogenic HIV or HTLV-1 protein epitopes (or to epitopes of any other infectious agent that induces autoreactive immune responses), would be an important and novel mode of preventing infectious tissue damage.

The ability to induce tolerance to an immune response induced by an infectious agent to prevent tissue destruction has been proposed as a method of treatment of Herpes simplex virus (HSV-1) corneal inflammation (R.L. Hendricks, et al, J. Immunol. 142:263-269, 1989).

The form of antigen has been suggested to be important regarding determination of whether a protein antigen is an immunogen or a toleragen

(Reviewed in Weigle (1989) The role of the physical state of human gamma globulin in the in vivo and in vitro induction of immunological tolerance. Chapter 5G, Vol. II, p 51-57). Whereas
5 high molecular weight aggregated gamma globulin is a potent immunogen, low molecular weight globulin is a toleragen (W.O. Weigle, Chpt. 5G, Vol. II, p.51-57, 1989). In this case, the ability of aggregated gamma globulin to induce endogenous IL1
10 has been suggested as the mode of immunogenicity of aggregated gamma globulin (L.C. Gahring, et al, J. Immunol. 145:1318-1323, 1990)

Others have suggested that some T cell epitopes are inherently immunogenic and some are
15 toleragenic (D.R. Milich, et al, J. Immunol. 143:3148-3156, 1989). Milich has converted toleragenic epitopes of Hepatitis B core antigen to immunogenic epitopes by single amino acid substitutions in the T cell epitopes (D.R. Milich,
20 et al, J. Immunol. 143:3148-3156, 1989). Benacerraf has suggested that freely diffusible antigens are toleragens whereas particulate antigens that are concentrated in cells of the reticuloendothelial system are immunogenic
25 (Benacerraf, B. Properties of antigens in relation to responsiveness and non-responsiveness, in Immunological Tolerance, M. Landy, W. Braun, Eds. Academic Press, NY, NY 1969). In contrast, Nossal reported that the particulate polymeric antigen
30 flagellin was a potent toleragen, and induced tolerance to antibody responses to the Salmonella flagella when injected into neonatal rats (Nossal, G Antigen Dosage in Relation to Responsiveness and Non-responsiveness, in Immunological Tolerance, M.
35 Landy, W. Braun, Eds. Academic Press, NY, NY 1969). Finally, immunogenicity versus toleragenicity of antigens has been proposed to be

due to their affinity of binding to MHC and TCR molecules (rev. in Spent et al, Science 248:1357-2363, 1990).

5 The present invention provides a method of modification of peptide immunogens whereby the modification changes a potent immunogen into a potent toleragen. The invention is based on the unexpected observation that the F-domain of HIV-1 gp41 confers to an antigen the ability to be a toleragen. Specifically, the hydrophobic N-terminal 12 amino acids of the gp41 envelope protein that mediate fusion of HIV to uninfected cells, the fusogenic (F) domain (M.L. Bosch, et al, Science, 244:694-697, 1989), were added C-terminal to the highly immunogenic T1-SP10 and T1-SP10(A) peptides (Table 3) (T.J. Palker, et al, J. Immunol. 142:3612-3619; M.K. Hart, et al, J. Immunol. 145:2677-2685, 1990; M.K. Hart, et al, Proc. Natl. Acad. Sci. USA 88:9448-9452, 1991).
10 When used as an immunogen in chimpanzees, the T1-SP10IIIB and the T1-SP10IIIB(A) peptides were potent immunogens, whereas the F-T1-SP10IIIB(A) peptide (M.K. Hart, et al, Proc. Natl. Acad. Sci. USA 88:9448-9452, 1991) were not as immunogenic at either low (.1mg/kg) or at high (.5 mg/kg) doses. Moreover, challenge of the animals with the highly immunogenic T1-SP10IIIB(A) peptide at month 16 of the immunization schedule proved that the F-T1-SP10IIIB(A) immunized animals were tolerant to the
20 T1-SP10IIIB(A) HIV gp120 env determinants.
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SUMMARY OF THE INVENTION

It is a general object of this invention to provide a method of inducing tolerance in a mammal to an immunogenic peptide or protein.

It is a specific object of this invention to provide a method of inducing tolerance in a mammal to an immunogenic peptide or protein comprising administering to the mammal a synthetic toleragen comprising a hydrophobic peptide linked to the N-terminus or C-terminus of the immunogenic peptide or protein, under conditions such that the tolerance is induced.

Further objects and advantages of the present invention will be clear from the description that follows.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Antibody Titers in ELISA Assay Against Immunizing Peptide Over Time In Chimpanzees Immunized with HIV Env Synthetic Peptides.

Figure 2. Peripheral Blood Mononuclear Cell Proliferative Responses to the T1-SP10IIIIB(A) Peptide in 7 Day Tritiated Thymidine Incorporation Assays.

Figure 3. PBMC Proliferative Responses of Chimpanzees Immunized with T1-SP10 Peptides and F-T1-SP10 Peptides to PHA.

Figure 4. Elution Profile of SP10MN(A) Over a G-75 Sephadex Column.

Figure 5. Elution of T1-SP10MN(A) Over a G-75 Sephadex Column.

Figure 6. Elution of F-T1-SP10MN(A) Over a Sephadex G-75 Column.

Figure 7. Elution of F-SP10MN(A) Over G-75 Column.

Figure 8. Results of DSP Cross-linking Analysis Using F-T1-SP10IIIIB(A) Peptide.

Figure 9. Hypothetical Model of F-T1-SP10IIIIB(A) in Aqueous Solution.

Figure 10. Variants of T1-SP10 peptides derived from HIV MN and IIIB Envelope Sequences.

Figure 11. Time course of PBMC 3H-thymidine incorporation responses to HIV Th-B peptide, T1-SP10IIIB(A), in chimpanzees immunized with HIV envelope synthetic peptides. Animals 884 (Figure 1A) and 1028 (Figure 1B) received the Th-B peptide, T1-SP10IIIB, initially (months 1-5), then the Th-B peptide, T-SP10IIIB(A) (month 6-8). After a boost with the Th-B peptide T-SP10IIIB(A) at month 14, both animals 884 and 1028 were immunized with the HIVMN Th-B peptide, T-SP10MN(A). Panels C and D show the responses of animal 1045 (Panel C) and 1070 (Panel D) to the HIVIIIB F-Th-B peptide (month 1-14), HIVIIIB Th-B peptide (month 16) and HIVMN Th-B peptide (months 17-19). All immunizations were with the indicated peptide in IFA, except all immunizations for animal 1028 after month 4, which were with peptides in PBS alone. Solid lines show data for peak proliferative responses (Δ cpm) to a wide dose range of HIVIIIB Th-B peptide. Dotted lines indicate peak proliferative response (Δ cpm) to a wide dose range of the HIVMN Th-B peptide.

Figure 12. Time course of PBMC 3H-thymidine incorporation response to PHA in chimpanzees immunized with HIV envelope synthetic peptides. Immunizations and chimpanzees as in Figure 11.

Figure 13. Time course of PBMC 3H-thymidine incorporation response to candida antigen in chimpanzees immunized with HIV envelope synthetic peptides. Immunizations and chimpanzees as in Figure 11.

Figure 14. Time course of absolute numbers of lymphocytes and lymphocytic subsets in chimpanzees immunized with HIV envelope synthetic

peptides. Immunizations and chimpanzees as in Figure 11. Points represent cell number/mm³ of peripheral blood lymphocytes and lymphocyte subsets. The elevated cell numbers in animal 1028 at month 4 coincided with an abscess at the injection sites.

Figure 15. HIV envelope hybrid synthetic peptides induced anti-HIV neutralizing antibodies in goats. Goat 102A was immunized with 3 mg of the F-Th-B peptide, F-T1-SP10IIIB(A) and goat 104A was immunized with the HIVIIIB Th-B peptide, T1-SP10IIIB. Immunizations were in CFA (first dose) and IFA (doses 2-4). Neutralizing titers are titers at which reverse transcriptase production was inhibited by 90% or greater.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a procedure whereby protein immunogens are derivatized by either synthesizing a hydrophobic amino acid sequence of 2 to 20 amino acids in length, N-terminal to the immunogenic protein or protein fragment, or covalently linking a hydrophobic peptide fragment of 2 to 20 amino acids in length N-terminal to the immunogenic protein or protein fragment, to yield an immunogen capable of inducing tolerance to the protein immunogen when administered to a mammal such as a primate (more preferably, humans).

In a preferred embodiment, the hydrophobic peptide is 5 to 15 amino acids in length. In yet another preferred embodiment, the hydrophobic peptide is 7 to 13 amino acids in length. In a further embodiment, the length of the hydrophobic peptide is 7, 8, 9, 10, 11, 12, or 13 amino acids in length. In yet another

embodiment, the hydrophobic peptide is at least 5 amino acids in length (more preferably, at least 10 amino acids in length).

Alternatively, immunogenic proteins known to be the targets of autoantibody or auto-T cell responses can be constructed using recombinant DNA technology to form new toleragens containing hydrophobic N-terminal regions as described above. While an advantageous construction of the invention is for the hydrophobic sequence to be N-terminal to the B or T cell epitope, in certain circumstances it may be advantageous to have the hydrophobic sequence C-terminal to the B or T cell epitope.

The hydrophobic region can be a fusion protein from HIV or HIV-related viruses (see Table 5), or can be a hydrophobic sequence of amino acids that is either randomly selected or is from a non-HIV related protein.

An example of this invention for inducing tolerance to antibodies against autoantigens is for the treatment of myasthenia gravis, whereby the F-sequence is synthesized N-terminal to the main immunogenic region of the acetylcholine receptor, WNPADYGGIK or WNPDDYGGVK (I. Papdoulis, et al, Biochem. J. 269:239-245, 1990). The resulting immunogen is AVGIGALFLGFLWNPADYGGIK or AVGIGALFLGFLWNPDDYGGVK.

Another example of a B cell toleragen is a hybrid protein comprising the HIV fusion domain synthesized either linearly N-terminal to B cell peptide epitopes of the insulin molecule or covalently linked to the whole insulin molecule or covalently linked or constructed using recombinant DNA techniques to a peptide insulin fragment or to the whole insulin molecule. The resulting immunogen is AVGIGALFLGFL-insulin or AVGIGALFLGFL-

insulin p ptide fragment. These types of toleragens can be used to prevent the onset of juvenile diabetes mellitus, (J.P. Palmer, et al, Science 222:1337-1339, 1983; B.M. Dean, et al, Diabetologia 23:339-342, 1986) and to treat patients with insulin antibodies in the setting of insulin resulin resistance (J.D. Schnatz, Dolovich, et al, J. Allergy, 46:127-1137, 1970).

Another example of a B cell toleragen is a hybrid protein comprising the HIV fusion domain synthesized either linearly N-terminal to B cell peptide epitopes of the TSH receptor molecule or covalently linked to the whole TSH receptor molecule or covalently linked or constructed using recombinant DNA techniques to a peptide TSH receptor fragment or to the whole TSH receptor molecule. The resulting immunogen is AVGIGALFLGFL-TSH receptor or AVGIGALFLGFL-TSH receptor peptide fragment. These types of toleragens can be used to treat autoimmune thyroid disease (Graves' Disease) (T. Mori, et al, Biochem. & Biophy. Res. Comm. 178:165-172, 1991; M. Murakami, et al, Biochem. & Biophy. Res. Comm. 171:512-518, 1990). Table 6 summarizes B cell epitopes on the thyrotropin (TSH) receptor to which Graves' patient sera bind (T. Mori, et al, Biochem. & Biophy. Res. Comm. 178:165-172, 1991; M. Murakami, et al, Biochem. & Biophy. Res. Comm. 171:512-518, 1990; O. Takai, et al, Biochem. & Biophy. Res. Comm. 179:319-326, 1991; T. Piraphatdis, et al, Biochem. & Biophy. Res. Comm. 172:529-536, 1990). Of interest is the sequence YVVFEEQEDEIIGF identified by 2 studies that inhibits the TSH activity of the autoantibodies (T. Mori, et al, Biochem. & Biophy. Res. Comm. 178:165-172, 1991; O. Takai, et al, Biochem. & Biophy. Res. Comm. 179:319-326, 1991).

Thus constructs for inducing tolerance to anti-TSH antibodies in Graves' disease are

AVGIGALFLGFLYVFFEEQEDEI or

AVGIGALFLGFLHQEEDFRVTCKDIQRIPSLPPSTQT or

5 AVGIGALFLGFLLRQRKSVNALNSPLHQEYEENLGDSIVGY or

AVGIGALFLGFLYYVFFEEQEDEIIGF or

AVGIGALFLGFLYKELPLLKFL.

An example of the use of this invention in the induction of tolerance to autoimmune T cell antigens is a hybrid protein comprised of the HIV fusion domain synthesized either linearly N-terminal to T cell peptide epitopes of the myelin basic protein molecule or covalently linked or constructed using recombinant DNA techniques to a myelin protein molecule. The resulting immunogen is AVGIGALFLGFL-myelin basic protein or AVGIGALFLGFL-myelin basic protein peptide fragment. In the case of the myelin basic protein peptide fragment, the encephalitogenic T cell epitopes are known, one of which is contained in sequence 69-89 of bovine myelin basic protein (H. Offner, et al, J. Immunol. 141:3828-3832, 1988). In this case, one formulation of the toleragen is AVGIGALFLGFLGSLPQKSQRSQDENPVVHF. These types of toleragens can be used to treat experimental autoimmune encephalomyelitis, which is thought to be an excellent model of human multiple sclerosis (K.W. Wucherpfenning, et al, Immunol. Today, 12:277-281, 1991). When the specific epitopes are identified that are the T cell targets in multiple sclerosis, then those sequences can be substituted in the peptide above, and used to tolerize T cells to the pathogenic T cell epitope of whatever the protein antigen turns out to be involved in multiple sclerosis.

Another example of this invention for induction of tolerance to autoimmune T cell

antigens is a hybrid protein comprising the HIV fusion domain synthesized either linearly N-terminal to T cell peptide epitopes of the retinal S protein molecule or covalently linked to the whole retinal S protein molecule or covalently linked or constructed using recombinant DNA techniques to retinal S antigen fragment or to the whole retinal S antigen molecule. The resulting immunogen is AVGIGALFLGFL-retinal S protein or AVGIGALFLGFL-retinal S protein peptide fragment. In the case of the retinal S protein peptide fragment, the pathogenic T cell epitopes are known, one of which is present in the sequence 1169-1191 of retinal S protein (H. Sanui, et al, Exp. Med., 169:1947-1960, 1989). In this case, one formulation of the toleragen is AVGIGALFLGFLPTARSVGAADGSSWEGVGVV. These types of toleragens can be used to treat experimental autoimmune retinouveitis, which is thought to be an excellent model of human inflammatory eye diseases such as Bechet's syndrome and idiopathic retinouveitis (H. Sanui, et al, Exp. Med., 169:1947-1960, 1989). When the specific epitopes are discovered that are the T cell targets in human inflammatory eye disease, then those sequences can be substituted in the peptide above, and used to tolerize T cells to the pathogenic T cell epitope of whatever the protein antigen turns out to be in human retinouveitis.

For the treatment of pathogenic immune responses induced by an infectious agent, an example of the invention is the treatment of HTLV-I associated myelopathy syndrome seen in tropical spastic paraparesis (rev. in Jacobson et al J. Immunol. 146:1155-1162, 1991). In this disease, there is strong evidence that the neurologic disease is caused by the induction of

cytotoxic T cells (CTL) against HTLV-I infected cells in the central nervous system (S. Jacobson, et al, J. Immunol. 146:1155-1162, 1991).

Jacobson, et al have shown that one primary region of HTLV-I env gp46 that induces CTL in tropical spastic paraparesis (TSP) is aa196-209 of gp46 as defined by peptide SP4a1 (S. Jacobson, et al, J. Immunol. 146:1155-1162, 1991; T.J. Palker, et al, J. Immunol., 142:971-978, 1989; A. Kurata, et al, J. Immunol., 143:2024-2030, 1989). Thus, to treat TSP, the present invention can be embodied by the hybrid peptide AVGIGALFLGFLLDHILEPSIPWKSKK. When new pathogenic CTL epitopes of HTLV-I are discovered, the therapeutic construct can be F-X where F is the hydrophobic sequence and X is the CTL epitope of the infectious agent.

The clinical manifestations of HIV have been postulated to be due to autoimmune responses induced by components of HIV that have sequence homology to human MHC Class I or Class II molecules (G.W. Hoffman, et al, Prac. Natl. Acad. Sci. USA 88:3060-3064, 1991; H. Wigzell, et al, FASEB J. p.2406-2410, 1991; H. Golding, et al, J. Clin. Invest. 83:1430-1435; F. Grassi, et al, J. Ex. Med., 174:53-62, 1991; J.A.T. Young, Nature, 333:215, 1988; H. Golding, et al, J. Exp. Med., 167:914-923, 1988). For the treatment of HIV infection, the present invention can comprise a series of hybrid peptides, each peptide containing an N-terminal hydrophobic peptide such as the HIV gp41 fusion domain (Table 5) and a C-terminal peptide from each of the regions of HIV env proteins bearing sequence homology MHC class I or class II molecules (G.W. Hoffman, et al, Prac. Natl. Acad. Sci. USA 88:3060-3064, 1991; H. Wigzell, et al, FASEB J. p.2406-2410, 1991; H. Golding, et al, J. Clin. Invest. 83:1430-1435; F.

Grassi, et al, J. Ex. Med., 174:53-62, 1991;
J.A.T. Young, Nature, 333:215, 1988; H. Golding,
et al, J. Exp. Med., 167:914-923, 1988) (Table 7).
Alternatively, it may be advantageous to treat HIV
5 infected individuals with F-X peptides where F is
a hydrophobic peptide such as the fusogenic domain
of HIV and X is a peptide fragment of HIV that is
immunogenic to T or B cells. In this situation, a
mixture of peptides would be used to inhibit
10 destructive anti-HIV immune responses that were
damaging host HIV-infected antigen-presenting
cells. Examples of this type of peptide are shown
in Table 3 and Figure 10, and were the peptides
used that tolerized chimpanzees in Figures 1 and 2
15 to both T1-SP10(A) determinants and to whole gp120
protein (Table 4).

The present invention is described in
further detail in the following non-limiting
Example.

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Example 1

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Antibody titers in ELISA assay against
immunizing peptide over time in chimpanzees
immunized with HIV env synthetic peptides are
shown in Figure 1. For animals 884 and 1028, the
peptide used in the ELISA assay was T1-SP10IIIB.
For animals 1045 and 1070, the peptide used in the
ELISA assay was F-T1-SP10IIIB(A). All
immunizations were in incomplete Freund's Adjuvant
(IFA) + PBS (1:1) except for animal 1028 that
developed IM abscesses after immunization no. 3,
and had one immunization held, then had all
subsequent immunizations in PBS only. As can be
seen, T1-SP10 peptides were excellent immunogens
in animals 884 and 1028, while T1-SP10 peptides
with the HIV gp41 fusion (F) domain synthesized N-

terminal to the T1-SP10 peptide did not induce antibody titers as high or as of long duration as did peptides without the F domain.

It is important to note that animals 1045 and 1070 were challenged at month 16 with the immunogen T1-SP10IIIB(A) that induced such good antibody titers in animals 884 and 1028. Animals 1045 and 1028 did not respond to T1-SP10IIIB(A) in IFA, thus demonstrating that they were tolerant to the T1-SP10(A) from their prior immunizations with F-T1-SP10IIIB(A) peptide. It is also important to note that while boost of 884 at week 14 gave a rise in titer to T1-SP10IIIB(A) peptide, boost of 1028 at the same time did not. Boost of 884 was with IFA, while boost of 1028 was with no adjuvant, but rather only PBS.

Peripheral blood mononuclear cell proliferative responses to the T1-SP10IIIB(A) peptide in 7 day tritiated thymidine incorporation assays is shown in Figure 2. T1-SP10IIIB and T1-SP10IIIB(A) peptides induced high levels of proliferation of circulating PBMC in animals 884 and 1028. These levels fell to non-detectable levels after a 6 month rest (month 14) but rose again in animals 884 and 1028. Proliferative responses in animal 1028 rose with each boost after the 6 month rest even though the immunizations were in PBS alone with no adjuvant. As with B cell response, animals 1045 and 1070, immunized with F-T1-SP10IIIB(A) peptide, did not proliferate to T1-SP10IIIB(A) peptide. When these latter two animals were immunized with the T1-SP10IIIB(A) peptide that was a good immunogen in 884 and 1028, neither of the animals 1045, 1070 developed a proliferative response to T1-SP10IIIB(A)--thus proving that the addition of the F-domain N-terminal to the T1-SP10 peptide created

a toleragen that tolerized animals 1045 and 1070 to the T1 and SP10 regions of gp120. As shown in Table 4, while animals 884 and 1028 both responded in proliferative assays to native gp120, animals 1045 and 1070 were tolerant to native gp120 as well as to immunizing peptides.

PBMC proliferative responses of chimpanzees immunized with T1-SP10 peptides and F-T1-SP10 peptides to PHA are shown in Figure 3. Data show that while animals 1045 and 1070 were tolerant to T1 and SP10 regions of HIV gp120, PBMC PHA responses in these animals throughout the immunization period were normal.

Similar results were obtained with peripheral blood mononuclear cell (PBMC) responses to candida antigen in 7 day in vitro stimulation assay (not shown). Thus, while specifically tolerant to T1 AND SP10(A), HIV env determinants, animal 1029 and 1045 were not generally immunosuppressed and could respond to candida to PHA stimulation in vitro.

The effect on peptide quaternary structure of placement of a hydrophobic sequence N-terminal to a T cell and/or a B cell determinant was examined. Using G-75 chromatography in aqueous buffers and crosslinking of peptide monomers using the heterobifunctional agent Dithiobis (succinimidylpropionate) (DSP), it was determined that addition of a hydrophobic sequence such as the fusion (F) domain of HIV or HIV-like retroviruses confers on the T1-SP10(A) or the SP10(A) peptide the ability to form high molecular weight aggregates, that are likely in the form of protein micelles.

An elution profile of SP10MN(A) over a G-75 Sephadex column is shown in Figure 4. 4mg of each peptide in 2ml 50mM Tris-HCl (pH 7.5)

containing 100mM KCl and 5% glycerol, was applied directly to a 90 x 1.6 cm column of Sephadex G-75 equilibrated with 50mM Tris-HCl (pH 7.5) containing 100mM KCl. The sizing column was calibrated with blue dextran (200,000), bovine serum albumin (66,000), bovine erythrocyte carbonic anhydrase (29,000), horse heart cytochrome C (12,400) and bovine lung aprotinin (6,500). The elution position of each peptide was determined by continuous measurement of eluent absorbance at OD 280. The corresponding molecular weight of each peptide peak was calculated from the calibration curve of the column. Each peptide was also applied to the column equilibrated with the same buffer containing 0.1% C12E9 [polyoxyethylene (9) lauryl ether]. The SP10MN(A) peptide (predicted Mr=2878) migrated as forms of 65 Da or lower. Similarly, the T1-SP10MN(A) (predicted Mr=4771) peptide also migrated as low mw forms ranging from 12,000 Da to 6,500 Da (Figure 5). In contrast, both the F-SP10MN(A) (Mr=4038) and the F-T1-SP10MN(A) peptides (Mr=5930) contained high molecular weight forms that migrated at ~66,000 Da (Figures 6 and 7). Methods used in Figures 5, 6, and 7 were as in Figure 4.

The results of DSP cross-linking analysis using F-T1-SP10IIIB(A) peptide are shown in Figure 8. Lane C shows the form of the peptide with no DSP added in PBS when run under non-reducing conditions in SDS-PAGE. Lanes D,E,F, and G show the effect on peptide MW when the peptide is cross-linked with 6.25µg (D), 12.5µg (E), 25µg (F) and 50µg (G) of DSP prior to SDS-PAGE. Lane H shows the results of addition of 2-ME to peptide cross-linked with 50µg of DSP and then run under reducing conditions in SDS-PAGE showing all of the

cross-linked forms seen in lane H and all the multiple forms seen in non-reduced, non-cross-linked peptide seen in lane C, were now reduced to two bands at 7000 kDa. At present, the nature of the two bands in this peptide under reducing conditions is unknown; these two bands can be purified by cutting the bands out of preparative gels and can be analyzed by mass spectroscopy and sequenced. Lanes A and B show the results of crosslinking F-T1-SP10IIIIB(A) peptide in the presence to Triton-X 100 1% and run under reducing (A) and non-reducing conditions (B). Data demonstrate that the apparent hydrophobic interactions holding the high MW complexes together are resistant to disruption by this detergent.

A hypothetical model of F-T1-SP10IIIIB(A) in aqueous solution is shown in Figure 9. The model shows protein micelle formation with the hydrophobic fusion domain (F) regions of the peptide in the core of the micelle with the hydrophilic V3 regions projecting outward.

Examples 2-8

The following experimental details and protocols are referenced in Examples 2-8.

Peptides: Peptides used in Examples 2-8 that follow are listed in Table 8. Peptide synthesis was performed using either t-boc or f-moc chemistry with a peptide synthesizer (A431; Applied Biosystems, Inc. Foster City, CA). Peptides were purified using HPLC, and the molecular weight was determined by fast atom bombardment mass spectrometry (R. B. Van Breeman, North Carolina State University, Raleigh, NC) using a double-focusing mass spectrometer

(HXIIIOHF; Joel Ltd., Tokyo, Japan). For Th-B and F-Th-B peptides (Table 8), expected molecular mass of F-Th-B peptide, F-T1-SP10IIIB(A), was 5908, observed was 5907; expected molecular weight of Th-B peptide, T1-SP10III.B, was 4061, observed was 4062; expected and observed molecular weight of Th-B peptide, T1-SP10IIIB(a) was 4,749, and expected and observed molecular weight of Th-B peptide, T1-SP10MN(A), was 4771. For the peptides used in the following Examples (Table 8), the peptide amounts are gross weights. The % water by Karl Fisher Test (Galbraith Laboratories, Inc. Knoxville, TN) for each peptide was F-T1-SP10IIIB(A), 6%; T1-SP10IIIB(A), 8%; T1-SP10IIIB, 6% and T1-SP10MN(A), 8%.

Animals: Chimpanzees were housed at the New Mexico State University Primate Facility at Alamogordo, NM. Chimpanzee No. 884 (15 yrs. old) and 1028 (12 yrs. old) had the same sire; animal 1045 (10 yrs. old) and 1070 (11 yrs. old) were unrelated to each other and to animals 884 and 1028. Outbred goats were housed at the Duke University Animal Facilities.

Immunizations: For goats, 3 mg of peptide were injected intramuscularly in each gluteal region in complete Freund's adjuvant (CFA) (1st dose), then incomplete Freund's adjuvant (IFA) (subsequent doses). For immunization of chimpanzees, varying doses of peptides were injected IM in IFA in a total volume of 4 cc, with 1 cc injected into right and left upper arms and thighs.

ELISA Assays: 2 μ g of Th-B peptide, T1-SP10IIIB, or rgp120IIIB (Repligen Corp., Cambridge, MD) in CBC buffer (15 mM Na_2CO_3 , 35 mM NaHCO_3 , pH9.6) was incubated overnight in each well of a 96 well flat bottom plate (Costar 3590).

Wells were blocked with CBC buffer supplemented with 3% bovine serum albumin (BSA) for at least 2 hrs and then were washed 3 times with PBS, 0.05% Tween 20. Primary antibody at various concentrations in serum diluent (95 ml PBS, 0.05% Tween 20, supplemented with 5 g BSA in 2 ml normal serum from same species as secondary antibody) was incubated for 90 min at 20°C. After washing three times, alkaline phosphatase-conjugated secondary antibody was added to each well (60 min at RT) and the plates washed. Substrate (1 mg/ml p-nitrophenyl phosphate, Sigma Chemical Co., St. Louis, MO) in 0.05M CBC-0.002M MgCl₂ was added to each well, and plates developed (60 min, 20°C) in the dark and read at 405 nm on an ELISA reader (Anthros; Denley Instruments Co., Durham, NC). Endpoint ELISA antibody titers were defined as the serum titer at which the experimental/control (E/C) OD value ≥ 3.0 .

HIV Neutralization Assays: The ability of chimpanzee or goat serum antibodies to neutralize HIV was determined in syncytium inhibition assay and reverse transcriptase inhibition assay as previously described (Palker et al, J. Immunol. 142:3612 (1989); Palker et al, Proc. Natl. Acad. Sci. USA 85:1932 (1988)). Sera were heat inactivated (30 min, 56°C) prior to each assay.

PBMC Isolation and In Vitro ³H-Thymidine Incorporation Assays: Chimpanzee or goat PBMC was isolated by standard density centrifugation techniques (Palker et al, J. Immunol. 142:3612 (1989); Haynes et al, Science 215:298 (1982)). In vitro assays of ³H-thymidine incorporation were performed as described (Palker et al, J. Immunol. 142:3612 (1989); Hart et al, J. Immunol. 145:2697 (1990)). For chimpanzee PBMC assays, in vitro

cultures were performed using 10% normal chimpanzee serum. Antigens used in PBMC proliferation were the Th-B peptides, T1SP10IIIB(A) and T1-SP10MN(A), (Table 8), and Candida albicans antigen (Greer Laboratories, Inc. Lenoir, NC). PHA (Burroughs Wellcome, Research Triangle Park, NC) was used in a wide dose range as a mitogen in 3 day PBMC ^3H -thymidine incorporation assays (Palker et al, J. Immunol. 142:3612 (1989); Hart et al, J. Immunol. 145:2697 (1990)). $\Delta\text{cpm} = \text{experimental cpm} - \text{control cpm}$.

Immunization Schedule: Because of previous studies demonstrating the immunogenicity of Th-B peptides in goats and rhesus monkeys (Hart et al, J. Immunol. 145:2697 (1990)), the initial comparison of peptide designs when this study began in 1989 was monthly injections of Th-B versus F-Th-B peptides (Table 8) at a dose of approximately 0.1 mg/kg (6 mg/animal). When neither peptide design induced neutralizing anti-HIVIIIB antibodies, the peptide doses were increased to approximately 0.5 mg/kg (30 mg/animal) and the right-hand side neutralizing sequence of HIVIIIB gp120 V3 loop (the (A) region) (Hart et al, Proc. Natl. Acad. Sci. USA 88:9448; Rusche et al Proc. Natl. Acad. Sci. USA 85:3198)) (Table 8) was added to the Th-B peptide to enhance the ability of this peptide to induce anti-HIVIIIB neutralizing antibodies. After 3 monthly injections with either ~0.5 mg/kg (30 mg) Th-B or F-Th-B peptide, the animals were rested for 6 months, and then reimmunized with either F-Th-B or Th-B with sequences from HIVIIIB, or with the Th-B peptide containing HIV env gp120 V3 sequences from the HIVMN isolate.

Flow Cytometry: Chimpanzee PB mononuclear cells were studied by standard flow

cytometry methods using a flow cytometer (751; Coulter Electronics, Inc., Hialeah, FL). PB lymphocytes were identified by the following markers; total T cells, CD3; T cell subunits, CD4 and CD8; B cells, CD19; and NK cells, CD56 and CD16.

Example 2

Immunogenicity of Th B and F Th B Peptides in Chimpanzees and Goats for Anti-Peptide and Anti-HIV gp 120 Antibody Responses

For chimpanzees immunized with HIVIIIB Th-B peptides (chimpanzee nos. 884 and 1028), antibody to immunizing peptide rose during the initial immunization period (Table 9). Chimpanzee no. 1028 developed an abscess at the immunization site, did not receive the month 5 immunization, and all subsequent immunizations after month 5 in animal 1028 were in PBS alone. Whereas peak end-point ELISA anti-peptide antibody titer at month 4 in animal 1028 was 1:819,200, antibody titers fell in animal 1028 after IFA was deleted from the immunogen, and remained low throughout the remainder of the immunization period (Table 9). In chimpanzee no. 884, antibody titers rose at month 7 to 1:204,800 after 5 immunizations with Th-B peptides. Continued immunization of animal 884 with high doses of Th-B peptide (30 mg/dose) resulted in no further increases in antibody titer (Table 9).

In contrast, anti-peptide antibody levels were much lower during months 1-10 of immunization of animals 1045 and 1070 with HIVIIIB F-Th-B peptide, with peak antibody levels against immunizing peptide of 1:25,600 and 1:12,800 at

month 7 for animals 1028 and 1070, respectively (Table 9). After a 6 month rest for all four animals, animals 884 and 1028 were immunized at month 14 with 6 mg of Th-B peptide. In chimpanzee no. 884, boosting with Th-B peptide in IFA at month 14 resulted in rise in titer of anti-peptide antibody to 1:102,400, while boosting of animal 1028 with peptide in PBS alone led to no antibody rise (Table 9).

In contrast, animals 1045 and 1070 were immunized at month 14 with 1 mg (~0.016 mg/kg) of F-Th-B to determine if the prior doses of F-Th-B peptide were excessive and induced high zone tolerance, and if smaller amounts of F-derivatized peptide would be more immunogenic. Immunization of both chimpanzee nos. 1045 and 1070 with 1 mg of F-Th-B peptide after a 6 month rest resulted in only minimal rises in serum titers of anti-peptide antibody to 1:800 (Table 9).

To determine if chimpanzees 1045 and 1070 were tolerant to Th-B peptides, both animals were immunized on month 16 with HIVIIIIB Th-B peptide, T1-SP10IIIIB(A). Both animals 1045 and 1070 responded minimally to boosting with Th-B peptide with an anti-peptide antibody responses to 1:1600 and 1:3200, respectively, demonstrating that animals 1045 and 1070 were hyporesponsive at month 16 to Th-B HIV env epitopes (Table 9).

Example 3

Immunization of Animals 1045 and 1070 with HIVMN Th-B Peptide Induced High Levels of Anti-peptide Antibodies

Using a previously described strategy of breaking B cell tolerance by immunization with an

immunogen that is different from, but structurally related to, the tolerogen (Weigle, Natural and Acquired Immunologic Unresponsiveness (1967) Chapter 4, pp. 57-151), animals 1045 and 1070 were next immunized with the HIVMN Th-B peptide. The TH-B peptide from HIVMN contained the same Th (T1) gp 120 sequence as the HIVIIIB Th-B peptide, but contained different B cell gp 120 V3 B cell epitope sequences than those in the HIVIIIB Th-B peptide (Table 8). After 2 immunizations with Th-B of HIVMN, beginning at month 17, both chimpanzee nos. 1045 and 1070 had prompt rises in titer of antibodies to HIVIIIB (Table 9) and to HIVMN Th-B peptide (not shown) to antibody levels that were higher than had previously been obtained during the prior 18 months of study. At month 20, endpoint ELISA titers to the HIVMN Th-B peptide were 1:102,400 for animal 1045 and 1:204,800 for animal 1070.

20

Example 4

Chimpanzee B Cell Antibody Responses
to Recombinant HIVIIIB gp120 During the
20-Month Immunization Course

Endpoint ELISA antibody titers against recombinant HIVIIIB gp 120 were determined for sera from months 4-7 and 16-20 to correlate peak anti-peptide antibody levels with anti-gp120 HIV envelope antibody levels. It was found that peak anti-gp120 antibody levels in chimpanzee nos. 884 and 1028 during months 4-7 were both 1:25,600, whereas peak titers to gp120 in animals 1045 and 1070 during the same period were 1:6,400 and 0, respectively. As with anti-peptide antibody levels, boosting after a 6 month rest with peptide

in PBS in chimpanzee 1028 did not boost anti-gp120 antib dies.

Boosting with F-Th-B peptide at month 14 and with HIVIII TH-B at month 16 in animals 1070 and 1045 resulted in minimal rises in anti-gp120 antibody titers by month 17 (to 1:12,800). In contrast, boosting chimpanzees 1045 and 1070 with HIVMN Th-B peptide at month 17 induced high levels of anti-gp120IIIB antibody in both animals (1:102,400 and 1:51,200, respectively) by month 20 that rose coincident with rises in levels of anti-peptide antibody.

Example 5

Induction of Anti-Peptide and Anti-gp120 PBMC Proliferative Responses by HIV Env Peptides

Whereas HIVIIIB Th-B peptides induced high levels ($>100,000$ Acpm/ 10^6 cells) of PBMC ^3H -thymidine incorporation (animals 884 and 1028) (Figures 11A and 11B) during months 1-8, F-Th-B peptide did not induce levels of ^3H -thymidine incorporation above $100,000$ Acpm/ 10^6 cells during the same period (Figures 11C and 11D). Immunization of animals 1045 and 1070 with Th-B peptide at month 16 did not induce the presence of circulating PBMC capable of proliferating to Th-B peptide in vitro (Figures 11C and 11D).

Interestingly, Th-B peptides at month 14-18 boosted PBMC proliferative responses in animal 1028, while anti-peptide antibody responses in animal 1028 during this time were not boosted (Figure 11B and Table 8).

Next, ^3H -thymidine incorporation of chimpanzee PBMC to either recombinant gp120IIIB or

to native gp120IIIB was tested. Table 4 shows the peak ³H-thymidine incorporation of chimpanzee PBMC to HIVIIIB gp120 for each animal during months 1-13, and demonstrates that neither chimpanzee no. 1070 nor 1045 (receiving F-Th-B peptide) had PBMC-proliferative responses to gp120 of greater than E/C>2 throughout the first 13 months of study. In contrast, animals 884 and 1028 (receiving Th-B peptides) did have anti-gp120 proliferative responses during the same period (Table 4).

To determine if PBMC proliferative responses to mitogenic or antigenic stimuli other than HIV immunogens were normal in the F-Th-B-immunized chimpanzees over the 20 months of study, we also measured PBMC proliferative responses to PHA were also measured (Figure 12) and to Candida (Figure 13). While peak PHA PBMC proliferative responses were nearly identical in the four chimpanzees, Candida PBMC-proliferative responses varied from animal to animal and from month to month. However, in animals 1045 and 1070, it was found that Candida responses were intermittently present during the time of immunization with F-Th-B peptide at levels that were similar to levels present before the immunizations were begun (Figures 13C and 13D).

Example 6

Characterization of PB Lymphocyte Subsets During Immunization of Chimpanzees With HIV Env Peptides

To determine if immunization with either HIV env peptide type had effects on the number of circulating chimpanzee T, B or NK cell populations, the absolute numbers of these cell

typ s were determined throughout the immunization period (Figure 14, Table 10). Whereas preimmunization (before) and postimmunization (during) lymphocyte levels in animals 884 and 1028 were not significantly different (Table 10), animal 1045 became relatively lymphogenic ($p < 0.001$) during the course of immunization with F-Th-B peptide with the lymphocyte count $650/\text{mm}^3$ at week 12, compared to preimmunization levels of 2815 and 2597 lymphocytes/ mm^3 in months 1 and 2, respectively (Figure 14C). Whereas T cell levels significantly dropped an average of 59% and 44% in chimpanzee nos. 1045 ($p > 0.001$) and 1070 ($p > 0.02$), respectively, during the immunization period, T cell levels did not significantly change in animals 884 and 1028 during the same time ($p > 0.1$) (Table 10). B and NK cell levels dropped significantly in animal 1045, but did not change in animals 1070, 884 and 1028 (Table 10). Taken together, these data demonstrated that immunization with the F-derivatized HIV env peptide induced decreases in absolute levels of circulating T cells in both animals 1045 and 1070, and in B and NK cell levels in animal 1045, whereas immunization of chimpanzee nos. 884 and 1028 with HIV Th-B env peptides lacking the F domain did not significantly affect circulating lymphocyte levels.

Example 7

Ability of HIVIIIB F-Th-B and Th-B Peptides to Induce Anti-HIVIIIB Neutralizing Antibodies in Goats

To determine if the F-Th-B peptide used in the initial phase of the chimpanzee

immunization protocol was immunogenic in another species, 3 mg of either F-Th-B or Th-B peptide were used to immunize goats three times over 2 months and then used to boost goats after an 8 month rest (Figure 15). It was found that after the fourth immunization, both peptides were capable of inducing serum anti-HIVIIIIB neutralizing antibodies (Figure 15), and capable of inducing high levels ($\geq 500,000$ Acpm/ 120^6 cells) of PBMC ^3H -thymidine incorporation in vitro to Th-B or F-Th-B peptides. In addition, serum endpoint ELISA titers of antibodies to immunizing peptide were the same in Th-B and F-Th-B-immunized goats. Thus, failure of the F-Th-B peptide to induce high levels of anti-peptide antibodies and PBMC-proliferative responses in chimpanzees was not due to lack of an inherent immunogenicity of the HIVIIIIB F-Th-B peptide, but rather was due to a specific effect of the F-derivatized peptide in chimpanzees.

Example 8

HIVMN Th-B Env Peptide Induced Anti-HIV Neutralizing Antibody in Chimpanzees

During the first 17 months of the immunization trial, serum-neutralizing antibodies against HIVIIIIB were always undetectable in syncytium inhibition assay and were $\leq 1:45$ in reverse transcriptase inhibition assay. However, following immunization of animals 1045 and 1070 at month 17 with HIVMN Th-B peptide, anti-HIV neutralizing antibodies were seen in syncytium inhibition assay (Table 11).

To determine why antibodies against HIVIIIIB Th-B peptides did not neutralize HIVIIIIB in vitro during the first 17 months of immunization, sera from the early peak anti-HIVIIIIB peptide antibody responses (month 6) were assayed for reactivity to the individual epitopes of the Th-B peptides. It was found that at the time of initial titers of anti-Th-B peptide responses, most of the antibody reactivity in sera from animals 884 and 1028 was indeed directed to the primary amino acid sequence of the neutralizing V3 loop region defined by the peptide (TRKSIRIQRGPCR) (Table 12,). These data indicate that antibodies made by chimpanzee nos. 884 and 1028 at 7 months after immunization with the HIVIIIIB Th-B HIV env peptides did not recognize the appropriate secondary V3 loop structure(s) necessary for neutralizing HIVIIIIB, although the animals did make antibody responses to the correct primary amino acid sequences of the neutralizing V3 B cell determinant of HIVIIIIB gp120.

Example 9

Regarding the induction of tolerance, additional clinical syndromes that might be treated using Fusion domain or Fusion domain-like peptides synthesized N- or C-terminal to an otherwise immunogenic antigen is in hypersensitivity to bee or wasp venom antigens and hypersensitivity to plant or animal allergens. The nucleotide and amino acid sequences of a number of allergens have now been synthesized, and those regions of the allergen proteins that induce IgE antibodies or T

helper cell responses that help to induce IgE responses are being mapped. Thus the primary structure of grass pollen (Silvanovich et al J. Biol. Chem. 266:1204-1220, 1991; Griffith et al 5 FEBS Letters 279:210-215, 1991; Perez et al J. Biol. Chem. 265:16210-16215, 1990; Singh et al Proc. Natl. Acad. Sci. USA 88:1384-1388, 1991), mite allergens (Tovey et al J. Exp. Med. 170:1457-1462, 1989; Yasel et al J. Immunol. 148:738-745 10 1992; Chua et al J. Exp. Med. 167:175-182, 1988; Chua et al Int. Arch. Allergy Appl. Immunol. 91:118-123, 1990), hornet venom (Fang, et al Proc. Natl Acad. Sci., USA 85:895-899, 1988), and tree pollen (Ebner et al J. Immunology 150:1047-1054, 15 1993; Jarolim et al Int. Arch. Allergy Appl. Immunol. 90:54-60, 1989; Valenta et al Science 253:557-560, 1991). For some of these allergen proteins, T cell epitopes have been mapped (Ebner et al J. Immunology 150:1047-1045, 1993) while for 20 others, likely T cell sites and hydrophilic B cell determinants can be predicted using computer algorithms (Kyte and Doolittle J. Mol. Biol. 157:105-132, 1982; Rothbard and Taylor EMBO J. 7:93, 1988; Margalit et al J. Immunol. 138:2213, 25 1987) and tested by synthesizing peptides and injecting animals, or by reacting patient serum antibodies or peripheral blood T cells with synthesized peptide in in vivo assays. Once

identified, T and B cell epitopes of bee, wasp or other allergens can be synthesized with a F domain or F-domain-like peptide N- or C-terminal to the allergenic T or B cell peptide, and the F-Allergen epitope hybrid peptide used to inject into patients that are sensitive to the allergen epitope. By this method, a patient can be made tolerant to the allergen epitope in the same manner as chimpanzees were made tolerant to T1-SP10 HIV env peptides by immunizing them with F-T1-SP10(A) peptide (Haynes et al J. Exp. Med. in press, 1993). Thus, in addition to treating autoimmune disease, F-derivatizing allergen T or B cell immunogenic peptides could product tolerogenic peptides for the treatment of allergic diseases.

A new technology has been developed whereby injection in vivo of cDNAs with a powerful promoter and encoding immunogenic peptides or proteins has been found to promote internalization and expression of cDNAs in host cells (Wolff et al Science 247:1465, 1990). Thus, the above strategy could be performed whereby cDNAs encoding F-derivatized peptides of autoantigens and/or allergens are injected instead of the peptides themselves, thus having the same effect as immunizing with peptides themselves. Moreover, the F-derivatized peptides and proteins could be

produced by recombinant DNA techniques instead of peptide synthesis of peptide synthesis and the same type of tolerizing immunogen obtained.

Another use of F domain- or F-like domain
5 derivatization of peptides and proteins is to confer upon the derivatized peptide or protein the ability to bind to the cell membrane and enter the cell. The fusion domain or a fusion-like domain could be conjugated to an RNA or DNA molecule as
10 well as a protein to promote entry into cells. The ability of a molecule to enter the cells is important for many molecules to act therapeutically, and can be overcome by addition of the F domain or an F-like domain to the
15 molecule that one wanted to get inside or cells. For example, a powerful inhibitor of cell activation would be a peptide, RNA or DNA species of molecule that competitively bound to an intracellular molecule necessary for cell
20 activation, but the peptide, RNA or DNA molecule itself did not activate or serve the normal function of the physiologic ligand that it was designed to mimic. Examples of peptide, RNA or DNA molecules that might inhibit cell activation
25 would be molecules that bound to intracellular tyrosine kinases, tyrosine phosphatases, protein Kinase C enzymes or G proteins, just to name a few examples. However, for peptide, RNA or DNA

inhibitory ligands to function as cell regulatory agents when administered as therapeutic agents, they must readily bind the cell without killing the cell, and be able to enter the cell and function intracellularly. It has been shown that F-derivatization of the T1-SP10IIIB(A) peptide with the HIV gp41 F domain promotes enhanced binding (Table 13) and entry (Table 14) of the derivatized T12-SP10IIIB(A) peptide into human B cells. This ability to promote entry of derivatized molecules into the inside of cells represents a novel drug delivery system with potential uses for delivering virtually any type of molecule (RNA, DNA, protein) inside cells for the desired therapeutic effect. For example, F-derivatized proteins of HIV regulatory proteins that might bind to viral RNA but not promote transcription of RNA thus preventing normal binding of HIV transcription factors might be used to treat HIV infections in vivo.

* * * * *

All publications mentioned hereinabove are hereby incorporated in their entirety by reference.

While the foregoing invention has been described in some detail for purposes of clarity

and understanding, it will be appreciated by one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope
5 of the invention and appended claims.

Table 1

Examples of Autoimmune Diseases or Disease Model Caused
By Autoreactive B Cell Responses

Disease	Pathogenic Antibody Specificity
Myasthenia Gravis (MG)	Anti-acetylcholine receptor antibodies cause weakness in MG
Juvenile Onset Diabetes Mellitus (Type 1 Diabetes)	Anti-insulin antibodies and anti-islet cell antibodies mediate islet cell destruction
Graves' Disease	Anti-thyroid stimulating hormone receptor antibodies mediate the disease
Insulin Resistance in Diabetes Mellitus	Anti-insulin antibodies prevent treatment of diabetes with insulin

Table 2

Examples Autoimmune Diseases or Disease Models Caused
by Autoreactive T Cell Responses

Experimental autoimmune uveoretinitis (EDU)	T cell responses against retinal S antigen cause eye damage
Experimental autoimmune encephalomyelitis (EAE)	T cell responses against myelin basic protein cause neuronal damage

Table 3

Peptide Sequences Used In Chimpanzee Immunizations	
F-T1-SP10IIIB(A)	AVGIGALFLGFLKQIINMWQEVGKAMYACTRPNNNTRKSIRIQRGPGRAFVTI
T1-SP10IIIB	KQIINMWQEVGKAMYACTRPNNNTRKSIRIQRGPG
T1SP10IIIB(A)	KQIINMWQEVGKAMYACTRPNNNTRKSIRIQRGPGRAFVTI

Table 4

Tritiated Thymidine Incorporation of Peripheral Blood Mononuclear
Cells Following In Vitro Stimulation With HIV Env gp120*

Chimpanzee No.	Immunogen	Pre-	Post-
		Immunization Δ cpm/ 10^6 cells (Post/Pre)	Immunization
884	T1-SP10IIIIB, then T1-SP10IIIIB(A)	169	39,189 (232)
1028	T1-SP10IIIIB, then T1-SP10IIIIB(A)	17,955	129,121 (7)
1045	F-T1-SP10IIIIB(A)	6,348	12,256 (2)
1070	F-T1-SP10IIIIB(A)	11,285	22,719 (2)

*Data represent the peak gp120 responses observed during the immunization period. Data for animals 884, 1028, and 1045 represent peak responses using from 2ug/ml to 0.5ug/ml of HIVIIIIB(LAI) recombinant gp120. Data for animal 1070 represent peak responses using from 1ug/ml to 0.5ug/ml of native HIVIIIIB(LAI) gp120.

Table 5

HIV Envelope gp41 Fusion Protein (F) Sequences From Multiple
HIV Isolates

Isolate	Sequence
<hr/>	
HIV-1	
BH10	A V G : I G A L F L G F L
MN	A A : : - - - - -
SC	- - - T - - - M - - - - -
SF2	- - - I V - - - M - - - - -
CDC4	- - - M L - - - M - - - - -
WMJ2	- - - T - - - M - - - - -
RF	- - - T - - - M - - - - -
ELI	- I - : L - - M - - - - -
MAL	- I - : L - - M - - - - -
Z6	- I - : L - - M - - - - -
Z321	- I - M : - - F - - - - -
JY1	- I - : L - - V - - - - -
WMJ-1	- - - A - - - M - - - - -
HIV-2	
ROD	R G V F V L G F L G F L
NIHZ	- - - - -
<hr/>	

Sequences for BH10 are aa 519-530 from Ratner, L, et al. Nature 313: 277-284, 1985. Sequences for the remainder of the HIV-1 and HIV-2 isolates from Myers, et al. Human Retroviruses and AIDS, 1988, Los Alamos National Laboratory, Los Alamos, New Mexico, p. II-90. WMJ-1 sequence from ref. 18.

Table 6

Regions of the TSH Receptor to Which Patient
Anti-TSH Receptor Autoantibodies Bind

Amino Acid No.	Sequence	Ref.
333-343	YVFFREQEDEI	17
12-36	HQEEDEFRTCKDIQRIPSLPPSTQT	18
289-317	LRQRKSVNALNSPLHQEYEENLGDSIVGY	18
352-366	YVFFEEQEDEIIGF	27
103-111	YKELPLLKFL	28

Amino acid numbers and sequence from the reference listed

Table 7

Examples of Hybrid Peptide Constructs That Could Be Used To
Treat Anti-HLA Immune Responses In AIDS

HIV gp120 homology with DP/DQ β chain

gp120 aa261-270 VVSTQLLLNG

HLA DP/DQ aa142-151 VVST*LI*NG

HIV gp41 homology with HLA DR β chain

gp41 aa837-844 EGTDRVI

HLA DR aa19-25 NGTERVR

Hybrid Immunogens: AVGIGALFLGFLVVSTQLLLNG

 AVGIGALFLGFLVVSTLING

 AVGIGALFLGFLEGTDRVI

 AVGIGALFLGFLNGTERVR

HIV gp120 and gp41 homologies with HLA Class II are from refs. 25
and 26.

TABLE 8

Sequences of Synthetic Peptide Constructs
Derived From HIV MN and HIVIIB Env gp120*

Peptide Name	Peptide Type	Peptide Composition and Sequence (Epitope Type)		
		F	T1(Th)	SP10(B cell) A(B cell)
F-T1-SP10IIB(A)	F-Th-B	AVGIGALFLGFLKQIINHMQEVGKAMHYACTRPNNTTRKSIRIQRPGRFVTI		
T1-SP10IIB(A)	Th-B	KQIINHMQEVGKAMHYACTRPNNTTRKSIRIQRPGRFVTI		
T1-SP10IIB	Th-B	KQIINHMQEVGKAMHYACTRPNNTTRKSIRIQRPGRFVTI		
T1-SP10MN(A)	Th-B	KQIINHMQEVGKAMHYACTRPNNTTRKSIRIQRPGRFVTI		

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*Each amino acid is represented by a single-letter code that is the first letter of its name, except for arginine (R), asparagine (N), glutamine (Q), glutamic acid (E), lysine (K), phenylalanine (F), tryptophan (W), tyrosine (Y), and aspartic acid (D). F (fusogenic domain) sequence is amino acids 519-530 from HIVIIB (27). T1 sequence is amino acids 428-443 from HIVIIB (27). SP10MN(A) sequence is amino acids 301-319 from HIVMN (28). SP10IIB sequence is amino acids 303-321 from HIVIIB. (A) sequence is amino acids 320-324 from HIVMN (28) and amino acids 322-327 from HIVIIB (27).

Th= T helper cell determinant.

B cell = B cell neutralizing antibody determinant.

A = Additional HIV gp120 V3 loop sequences added to the original synthetic peptide (SP10) sequence to add an additional neutralizing and CTL region to the HIV B cell determinant of the hybrid peptide.

27 = Ratner et al, Nature 313:277 (1985)

28 = Myers et al, Human Retroviruses and AIDS (1991), p. III 6-23

Table 19

Time Course of Anti-Peptide Antibody R spones in Chimpanzees
Immunized with HIV Envelope Synthetic Th-B or F-Th-B Peptides

Month of Study	Immunogen	Chimpanzee Number		Immunogen	Chimpanzee Number	
		884	1028		1045	1070
		Reciprocal of ELISA Titer			Reciprocal of ELISA Titer	
1		0	0		0	0
2	Th-B(IIIB) 6mg	0	0	F-Th-B(IIIB) 6mg	0	0
3	Th-B(IIIB) 6mg	51,200	102,400	F-Th-B(IIIB) 6mg	0	0
4	Th-B(IIIB) 6mg	25,600	819,200	F-Th-B(IIIB) 6mg	0	800
5	Th-B(IIIB) 6mg	25,600	204,800*	F-Th-B(IIIB) 6mg	1,600	200
6	Th-B(IIIB) 30mg	51,200	102,400	F-Th-B(IIIB) 30mg	25,600	12,800
7	Th-B(IIIB) 30mg	204,800	102,400	F-Th-B(IIIB) 30mg	25,600	12,800
8	Th-B(IIIB) 30mg	51,200	25,600	F-Th-B(IIIB) 30mg	6,400	12,800
9		51,200	51,200		3,200	6,400
10		12,800	25,600		800	800
11		51,200	25,600		800	1,600
12		51,200	25,600		1,600	800
13		25,600	25,600		200	200
14	Th-B(IIIB) 6mg	51,200	25,600	F-Th-B(IIIB) 1mg	200	400
15		102,400	12,800		800	800
16	Th-B(MN) 6mg	25,600	12,800	Th-B(IIIB) 6mg	100	0
17	Th-B(MN) 6mg	12,800	3,200	Th-B(MN) 6mg	1,600	3,200
18		25,600	6,400		6,400	25,600
19	Th-B(MN) 6mg	25,600	1,600	Th-B(MN) 6mg	6,400	51,200
20		51,200	6,400	Th-B(MN) 6mg	51,200	102,400#

Titers are endpoint ELISA titers (titers at which E/C were ≥ 3.0) against the Th-B peptide, T1-SP10IIIB.

* Animal 1028 did not receive the month 5 infection due to a sterile abscess at the injection site. All injections in animal 1028 after month 5 were in PBS alone.

Animal 1070 did not receive the month 20 immunization due to the presence of high levels of anti-HIV neutralize antibodies.

For animals 884 and 1028, immunizations at months 2-5 were with T1-SP10IIIB, months 6, 7, 8 and 14, T1-SP10IIIB(A). For animals 1045 and 1070 immunization at month 16 was with T1-SP10IIIB(A).

Table 10

Mean Lymphocyte and Lymphocyte Subset Levels in Chimpanzees
Before and During Immunization With HIV Envelope Synthetic Peptides.

Leukocyte Subset	Chimpanzee Number											
	884			1028			1043			1070		
	Before	During	% Change	Before	During	% Change	Before	During	% Change	Before	During	% Change
	Cells/mm ³ ± SEM											
Total												
Lymphocytes	4034±452	3046±249	-26%	3164±396	3286±660	+4%	3164±397	1426±116	-55%	3943±805	2768±296	-30%
T cells	2629±384	2054±178	-24%	2565±276	2027±402	-21%	2460±253	1012±82	-59%	3337±762	1887±184	-44%
B cells	356±47	365±39	+3%	411±103	458±47	+11%	293±32	175±15	-40%	302±53	232±22	-23%
NK cells	345±82	317±43	-9%	257±25	434±128	+68%	112±27	61±7	-45%	478±148	306±44	-36%

*"Before" samples were studied over a 3 month period prior to immunization with peptides; n = 5 for lymphocytes, n = 3 for T cells, B cells and NK cells. "During" samples were taken from months 2-14 of immunization; n = 11 for lymphocytes, T, B, and NK cells. Unless noted, p values for percent change comparing "before" values with "during" values was not significant with p > .05 using student's t test.

† = p > .001

‡ = p > .02

\$ = p > .005

Table 11

Neutralization of HIV LAI/IIIB and HIV MN in Syncytium Inhibition
Assay in Chimpanzees Immunized with T1-SP10 Peptides

Animal No.	Month 18		Month 19		Month 20	
	LAI/IIIB	MN	LAI/IIIB	MN	LAI/IIIB	MN
Presence of Neutralization in Syncytium Inhibition Assay (Reciprocal Titer in RT Inhibition Assay)						
884	-	- (20)	-	-	-	- (24)
1028	-	-	-	-	-	-
1045	-	- (23)	+/- (23)	- (23)	- (22)	- (24)
1070	+/- (92)	- (22)	+ (100)	+ (96)	+/- (86)	++ (350)

- = < 48% inhibition of syncytia.
 +/- = ≥ 49% and < 80% inhibition of syncytia.
 + = ≥ 80% inhibition of syncytia, titer 1:10.
 ++ = ≥ 80% inhibition of syncytia, titer 1:20.

Table 12

Reactivity of Chimpanzee Serum with Truncated Forms of
the Th-B Peptide T1-SP10IIIB*

Chimpanzee No. (Bleed Date)	Peptide Used in ELISA Binding Assay				
	T1-SP10IIIB	T1-flu	SP10C	SP10D	SP10E
	Endpoint Titer (> 3.0 E/C) In ELISA Assay				
884 (Month 7)	204,800	800	> 102,400*	51,200	3,200
1028 (Month 7)	102,400	800	102,400	51,200	3,200

*Peptides used in ELISA Assay were:

T1-SP10IIIB	-	KQIINMWQEVGKAMYACTRPNNTNRKSIRIQRGPG
T1-flu	-	KQIINMWQEVGKAMYATYQRTALVTG
SP10C	-	(C)TRKSIRIQRGPGR(Y)
SP10D	-	(C)IRIQRGPGR
SP10E	-	(C)TRPNNTNRKSIR

ELISA assay performed as described in Methods.

Flu sequence (TYQRTALVTG) is from influenza nucleoprotein, strain A PR/8/34 from Deres et al, Nature 342:561 (1989).

*E/C at 1:102,400 = 6.0.

Table 13

Effect of Derivatizing T1-SP10IIIB(A) Peptide With
the HIV gp41 Fusogenic (F) Domain on Peptide
Ability to Bind to Human Cells

Peptide	Antibody	MFC 4 Degrees C, 1 Hr.	MFC 37 Degrees C, 21 Hr.
None	Anti-gp120	7.6	13.6
T1-SP10IIIB(A) 10ug/ml	Anti-gp120	14.7	14.0
F1-T1-SP10IIIB(A)	Anti-gp120	82.8	36.7

Anti-gp120 monoclonal antibody was 0.5beta from the NIAID AIDS Research and Reference Reagent Program (Matsushita et al J. Virol. 62:2107, 1988). Cells used were human JY B cells which were incubated either for 1 hour at 4 degrees C or for 21 hours at 37 degrees C and then reacted with saturating amounts of the anti-gp120IIIB mab, 0.5beta followed by FITC-conjugated goat anti-mouse Ig reagent. The amount of fluorescence was determined on a flow cytometer and fluorescence brightness was expressed as MFC=mean channel fluorescence.

Table shows that conjugation of the F domain on the T1-SP10IIIB(A) peptide confers on it the ability to bind to JY B cells better than the T1-SP10IIIB(A) peptide alone, and that after incubation at 37 degrees C, the F-T1-SP10IIIB(A) peptide is decreased on the surface of the cells.

Table 14

Reactivity of anti-gp120 Monoclonal Antibody with
Acetone-Fixed JY B Cells That Had Been Incubated
With F-T1-SP10IIIB(A) Peptide (10 μ g/ml) For 21
Hours at 37 Degrees C

Peptide	Antibody	% Intracytoplasmic Positive
T1-SP10IIIB(A)	Control	0
T1-SP10IIIB(A)	Anti-gp120	0
F-T1-SP10IIIB(A)	Control	0
F-T1-Sp10IIIB(A)	Anti-gp120	76 faint, 24 bright

Cells were incubated as described in Table 13. After 21 hours at 37 degrees C, cytocentrifuge preparations of cells were prepared, acetone fixed, and reacted either with control mab P3x63 Ag8 or with anti-gp120 mab 0.5beta. Slides were read for either faint or bright intracytoplasmic fluorescence on a fluorescence microscope. Data show that after incubation of 10 ug/ml of peptide for 21 hours at 37 degrees C, the F-T1-SP10IIIB(A) peptide could be detected inside the JY B cells whereas the T1-SP10MN(A) peptide could not be detected.

WHAT IS CLAIMED IS:

1. A method of inducing immune tolerance in a mammal to an immunogenic peptide or protein comprising: administering to said mammal a synthetic immune system toleragen comprising a 2 to 20 amino acid hydrophobic peptide linked to the N-terminus or C-terminus of said immunogenic peptide or protein, under conditions such that said immune tolerance is induced.

2. The method according to claim 1, wherein said mammal is a primate.

3. The method according to claim 2, wherein said primate is a human.

4. The method according to claim 1, wherein the hydrophobic peptide comprises 5 to 15 amino acids.

5. The method according to claim 2, wherein the hydrophobic peptide comprises 7 to 13 amino acids.

6. The method according to claim 1, wherein the hydrophobic peptide is a segment from a HIV or HIV-related virus protein.

7. The method according to claim 1, wherein the hydrophobic peptide is AVGIGALFLGFL.

8. The method according to claim 1, wherein the immunogenic peptide or protein is an acetylcholine receptor protein.

9. The method according to claim 1, wherein the immunogenic peptide or protein is an acetylcholine receptor protein, or fragment thereof.

10. The method according to claim 1, wherein the immunogenic peptide or protein is an insulin protein, or fragment thereof.

11. The method according to claim 1, wherein the immunogenic peptide or protein is a TSH receptor protein, or fragment thereof.

12. The method according to claim 1, wherein the immunogenic peptide or protein is an autoimmune T cell antigen, or fragment thereof.

13. The method according to claim 1, wherein the immunogenic peptide or protein is a retinal S protein, or fragment thereof.

14. The method according to claim 1 wherein the immunogenic peptide or protein is a B cell determinant or a protein that induces pathogenic B cell antibody production in an autoimmune or inflammatory disease.

15. The method according to claim 1 wherein the hydrophobic part of the toleragen is any hydrophobic peptide from a transmembrane region of a transmembrane protein, or is a random mix of hydrophobic amino acids.

FIG. 1A

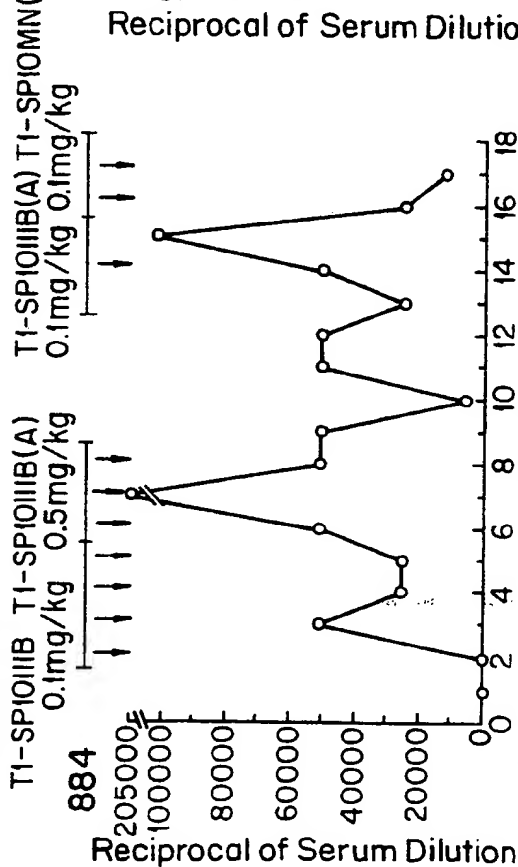


FIG. 1B

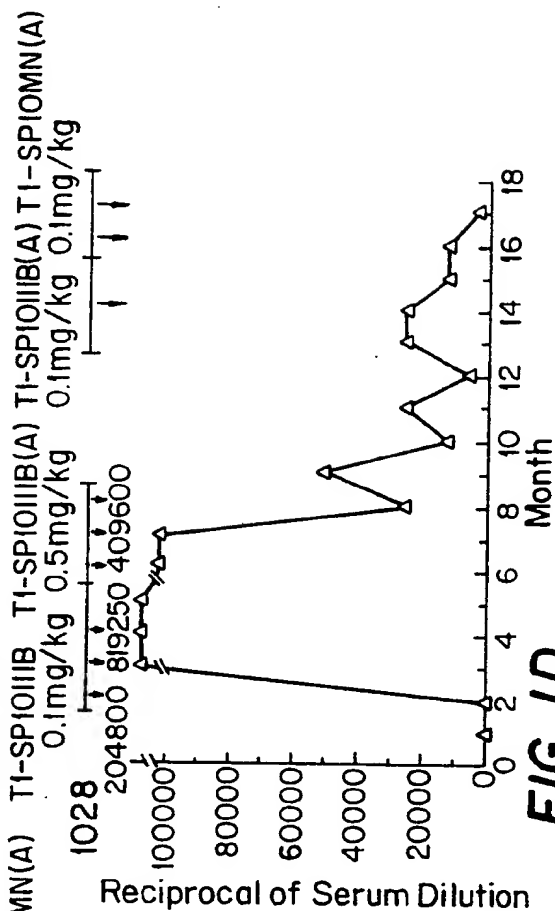


FIG. 1C

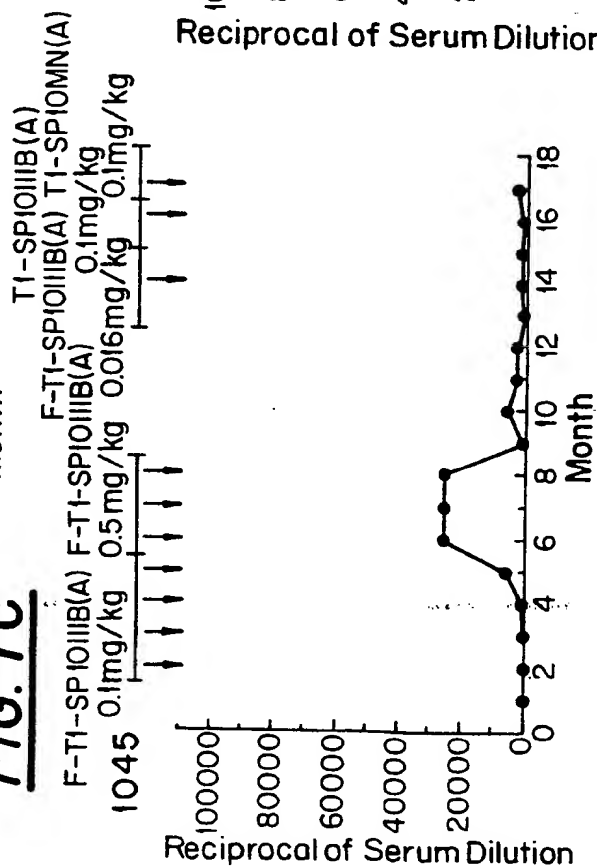


FIG. 1D

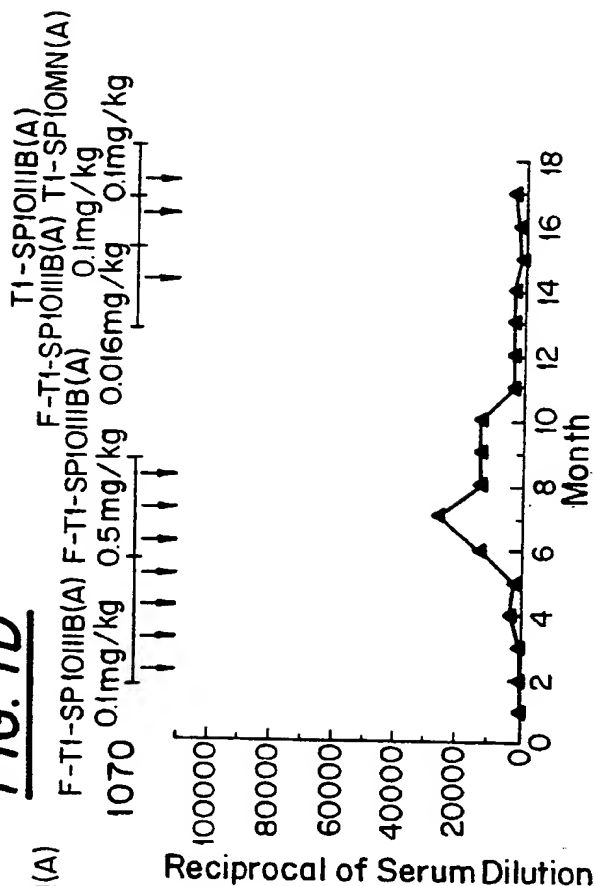


FIG. 2B

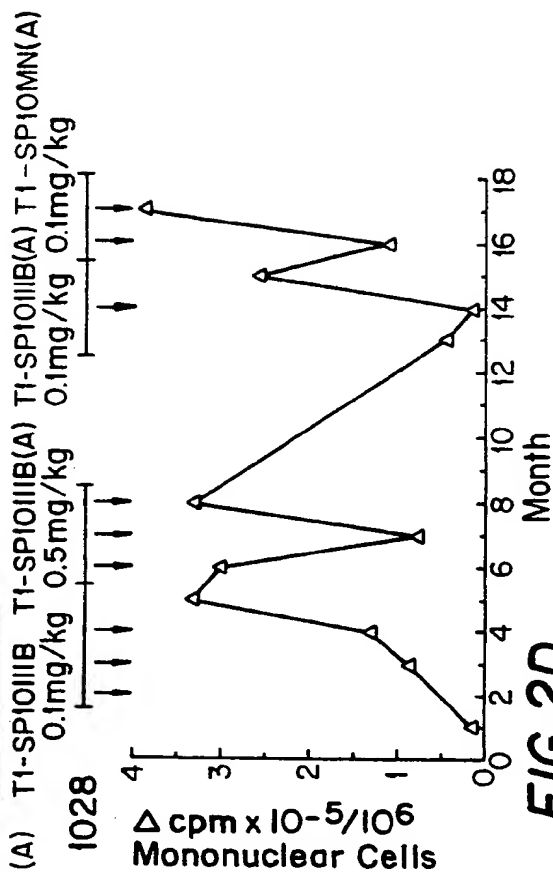


FIG. 2D

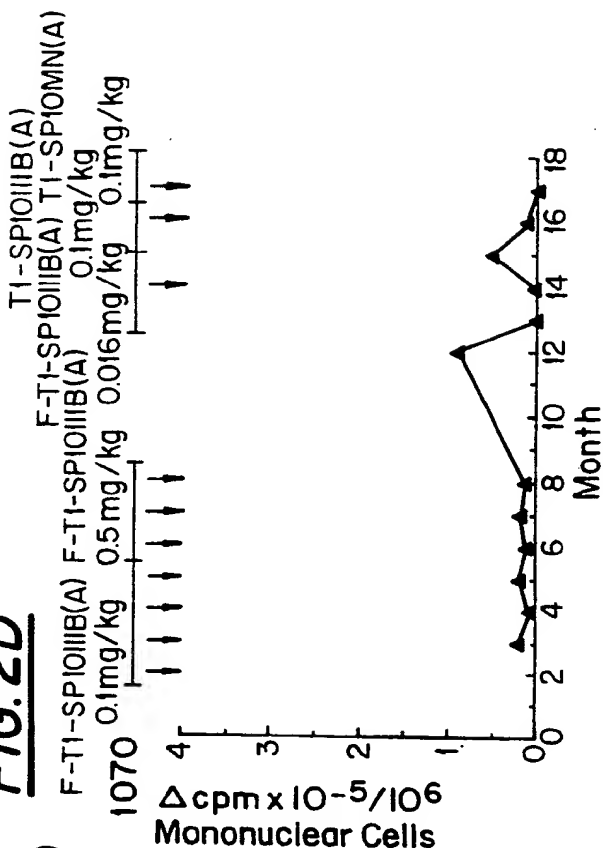


FIG. 2A

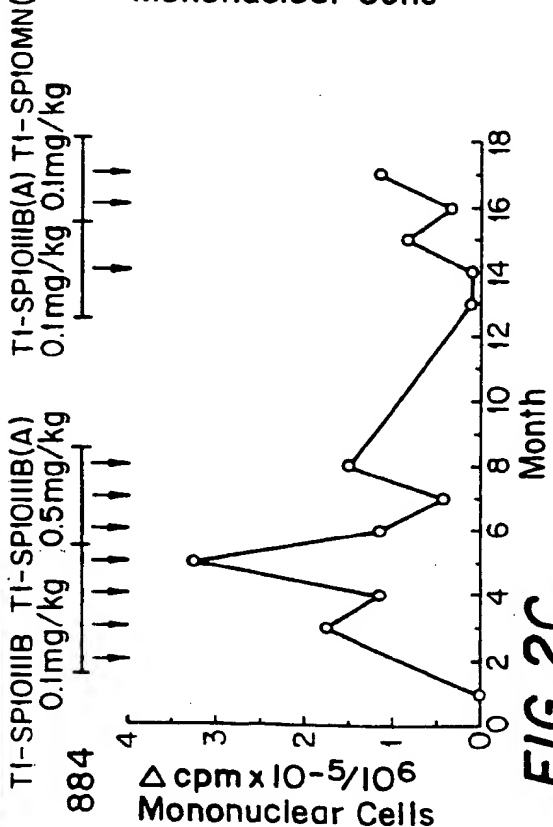


FIG. 2C

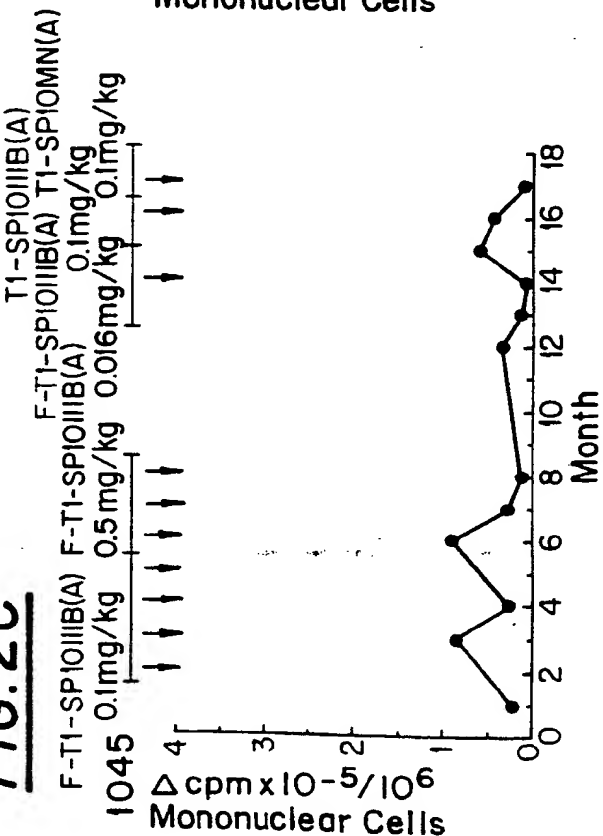


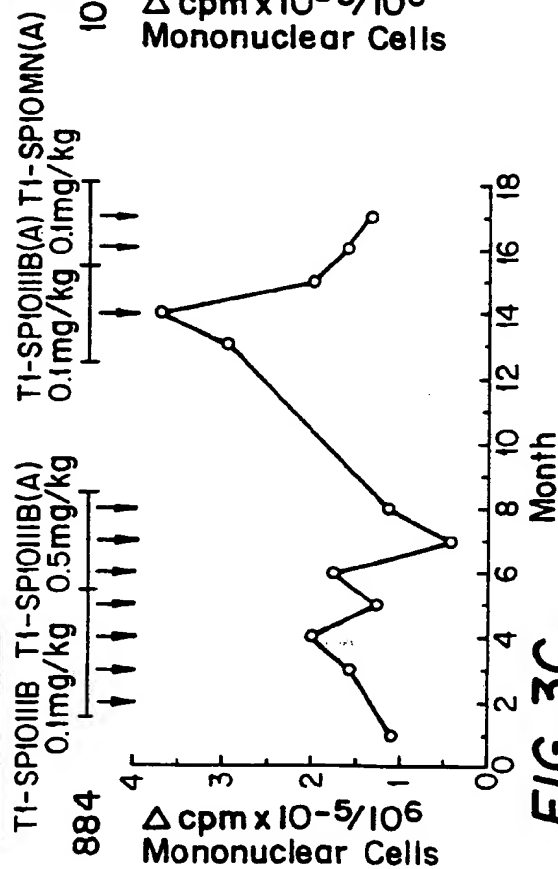
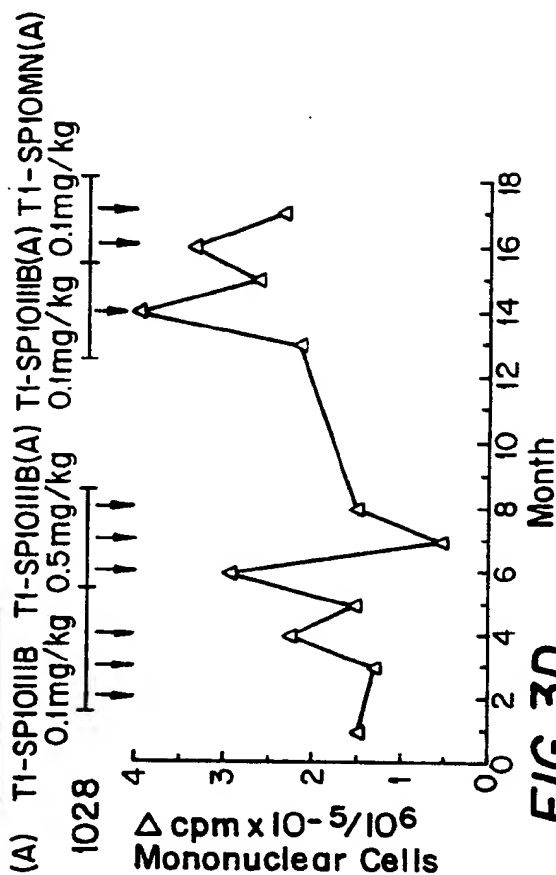
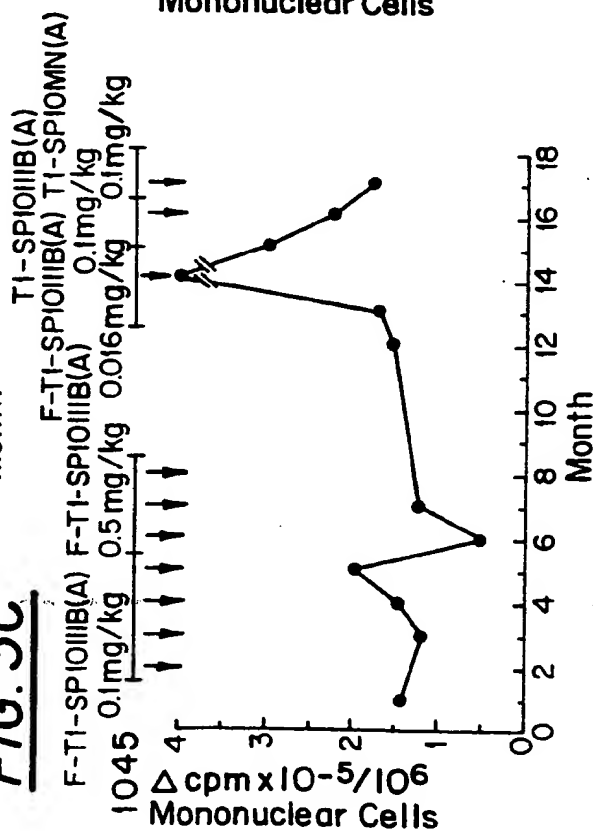
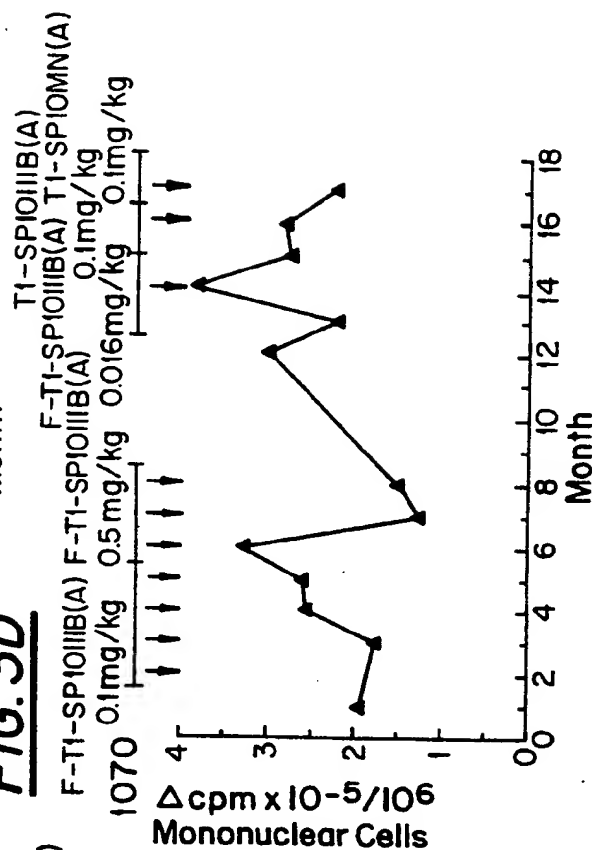
FIG. 3A**FIG. 3B****FIG. 3C****FIG. 3D**

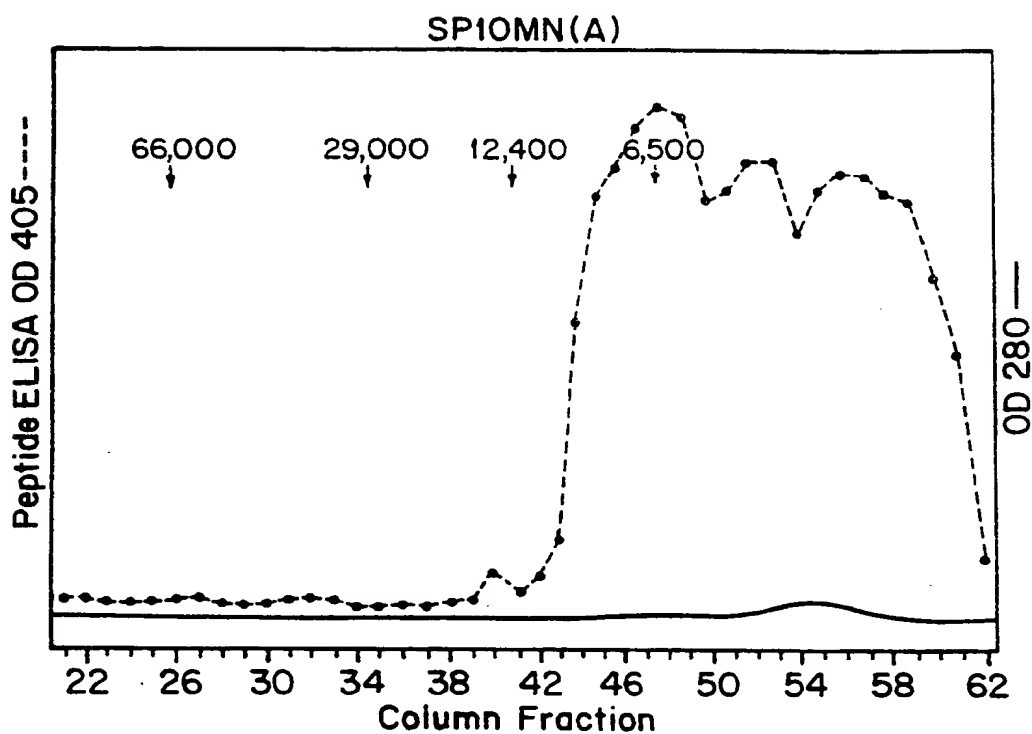
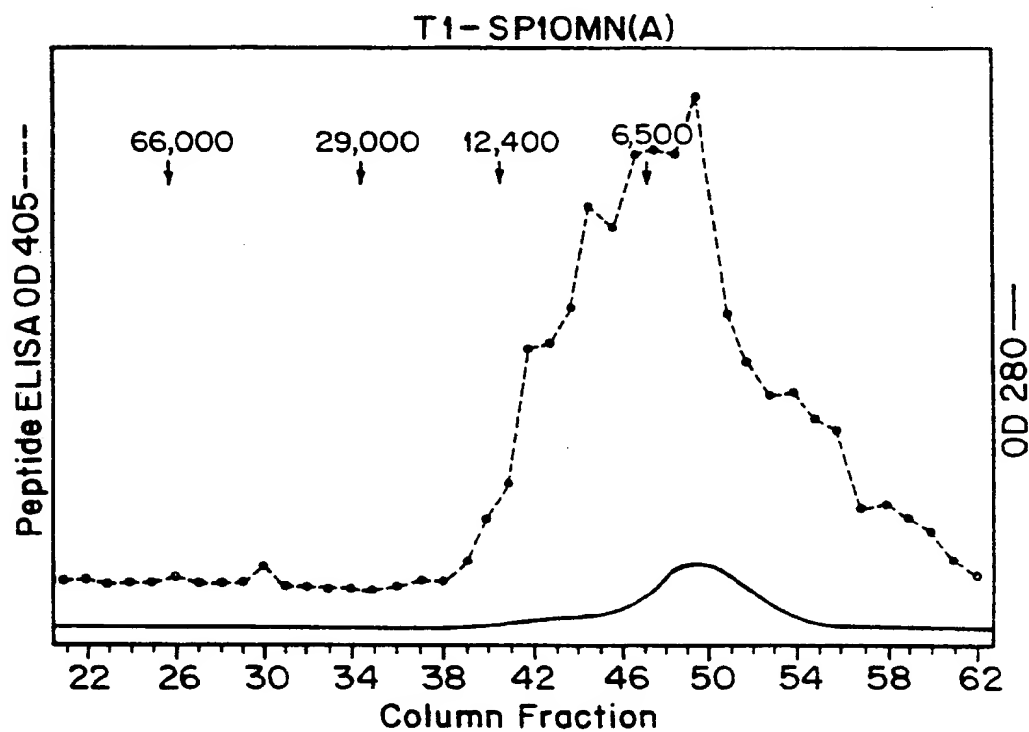
FIG. 4**FIG. 5**

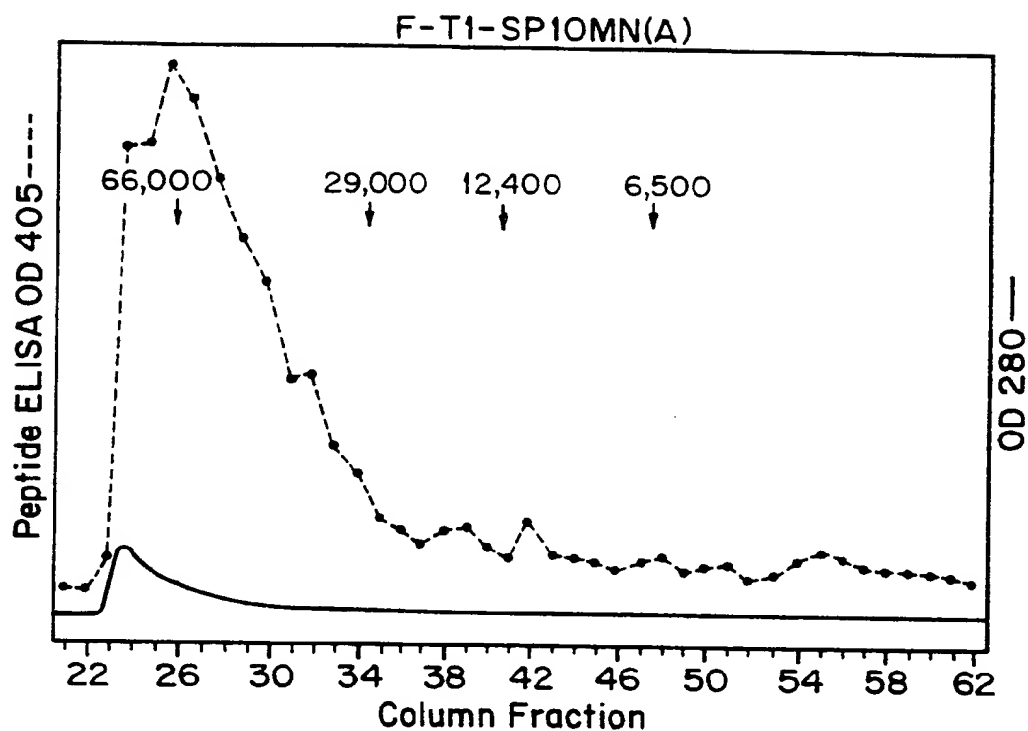
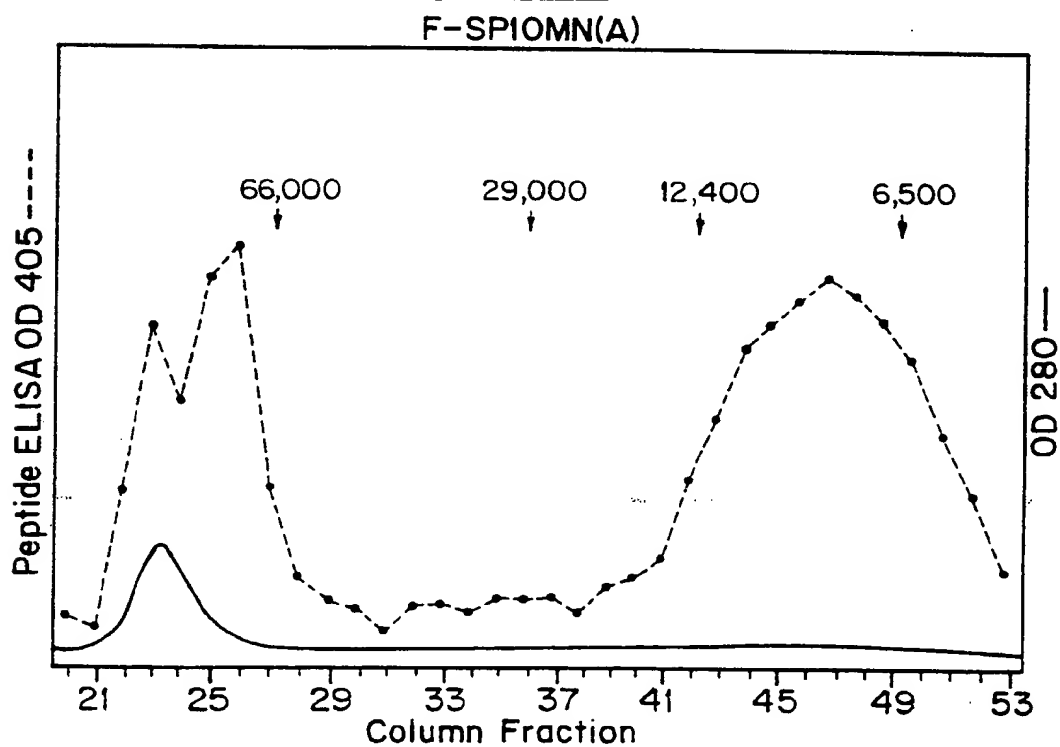
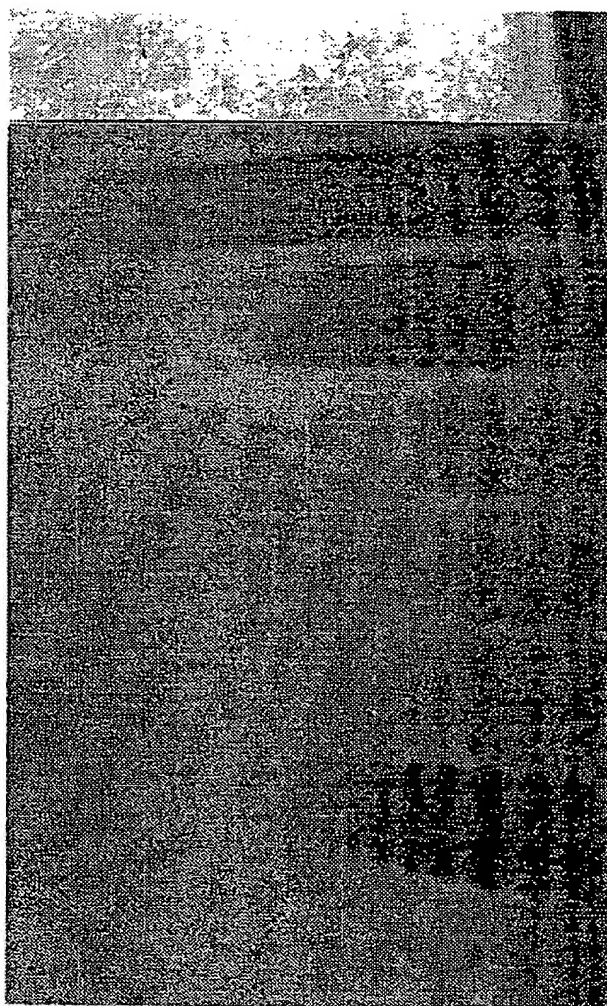
FIG. 6**FIG. 7**

FIG. 8

A B C D E F G H



110-
84-
47-
33-
24-
16-

SUBSTITUTE SHEET

FIG. 9

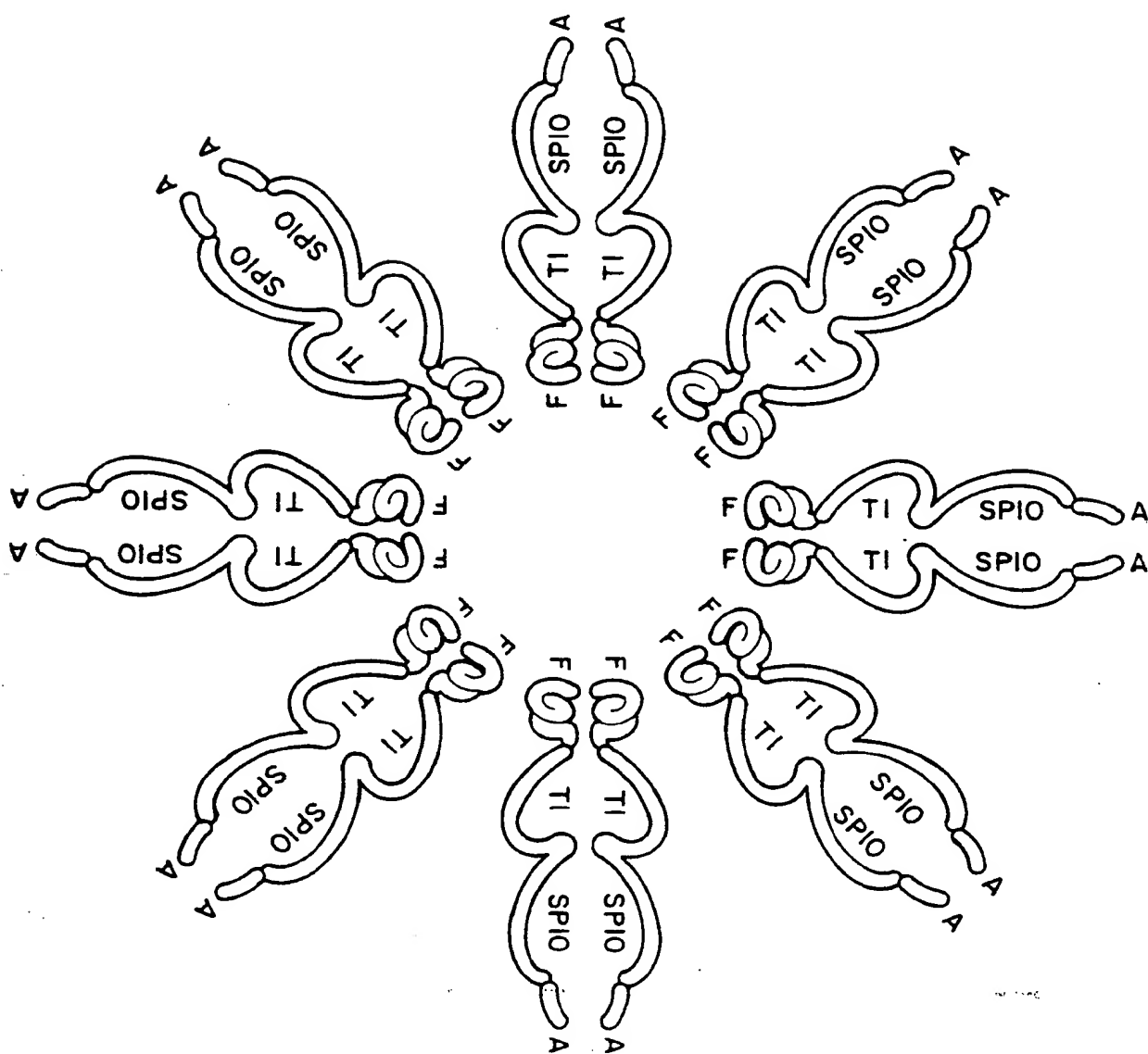


FIG. 10

Envelope Sequences

Peptide Name	Region
HIV MN	F T1 SP10 A
T1-SP10MN	KQIINMWQEVGKAMYACTRPNYNKRKRIHIGPGRA
T1-SP10MN (A)	KQIINMWQEVGKAMYACTRPNYNKRKRIHIGPGRA FYTTK
F-T1-SP10MN	AVGIGALFLGLKQIINMWQEVGKAMYACTRPNYNKRKRIHIGPGRA
F-T1-SP10MN (A)	AVGIGALFLGLKQIINMWQEVGKAMYACTRPNYNKRKRIHIGPGRA FYTTK

FIG. 10 cont.

F-SP10MN(A)	AVGIGALFLGFL.....CTRPYNKRKRRIHIGPGR FYTTK
SP10MN(A)	CTRPYNKRKRRIHIGPGR FYTTK
HIVIIIB	
T1-SP10IIIB(A)	KQIINMWQEVGKAMYACTRPNNNTRKSIRIQRGPG RAFVTI
F-T1- SP10IIIB(A)	AVGIGALFLGFLKQIINMWQEVGKAMYACTRPNNNTRKSIRIQRGPG RAFVTI

FIG. IIA

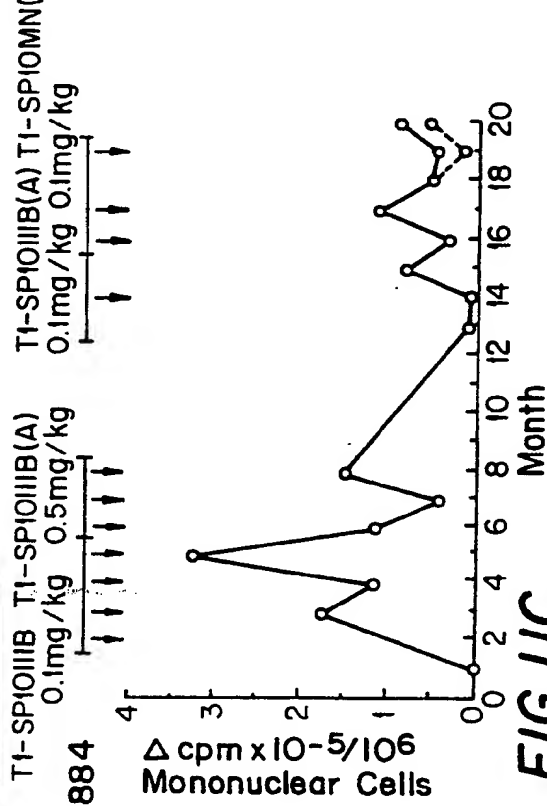


FIG. IIB

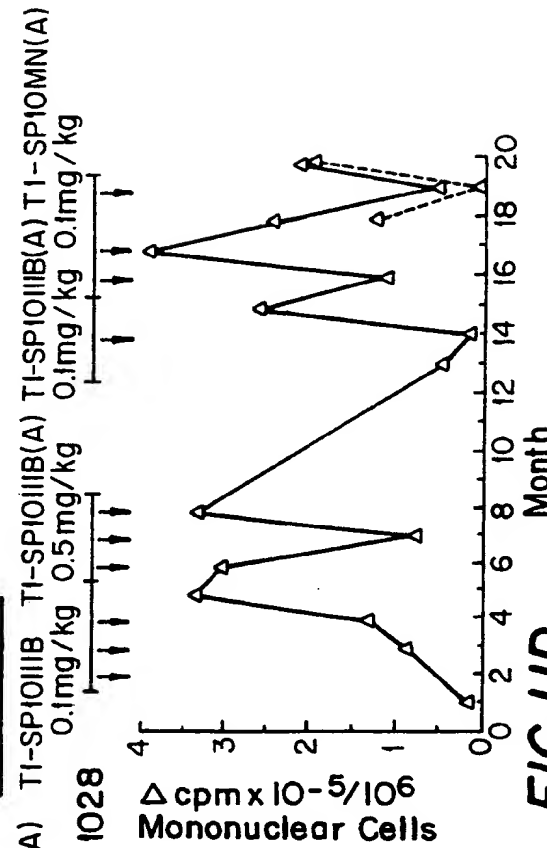


FIG. IIC

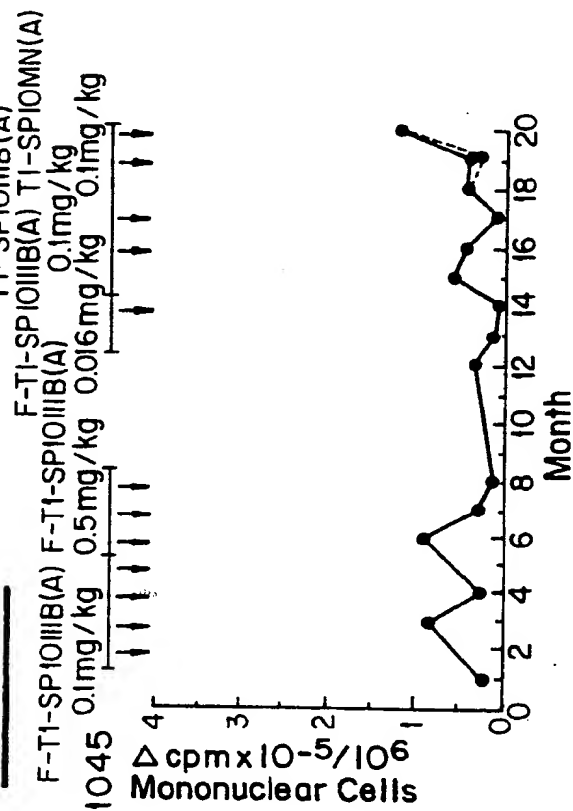


FIG. IID

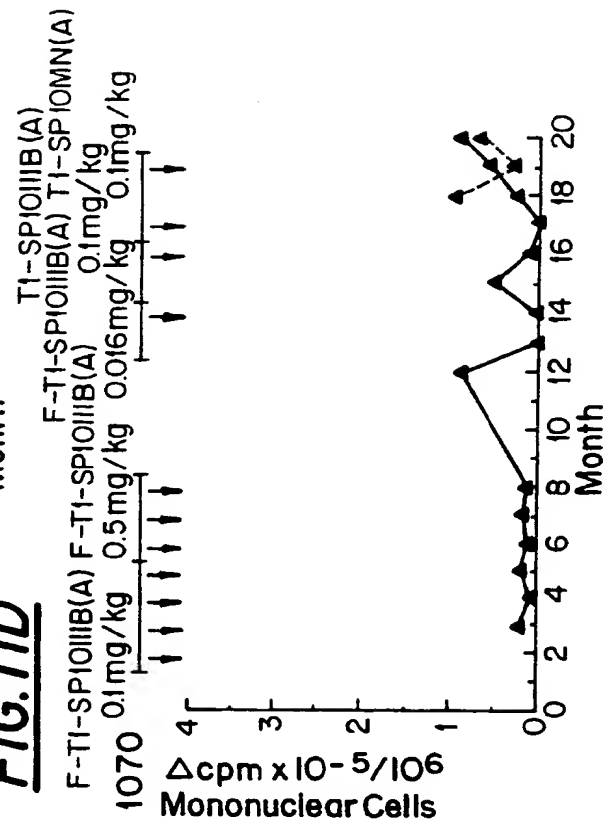


FIG. 12A

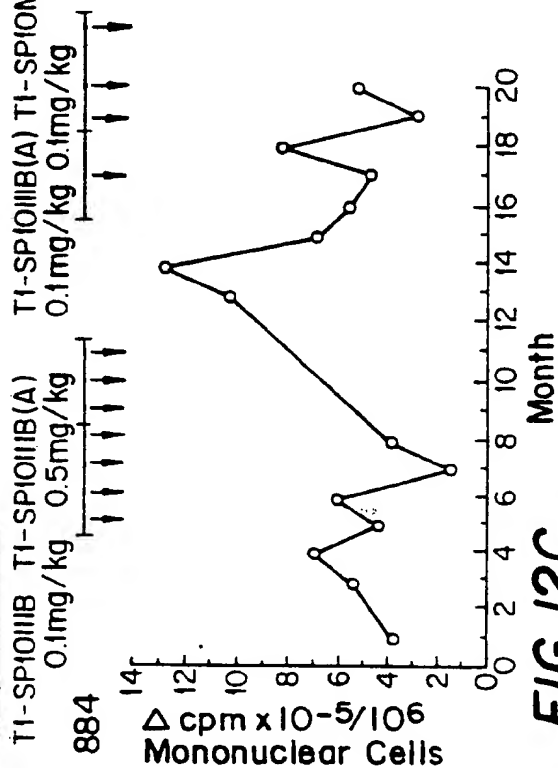


FIG. 12B

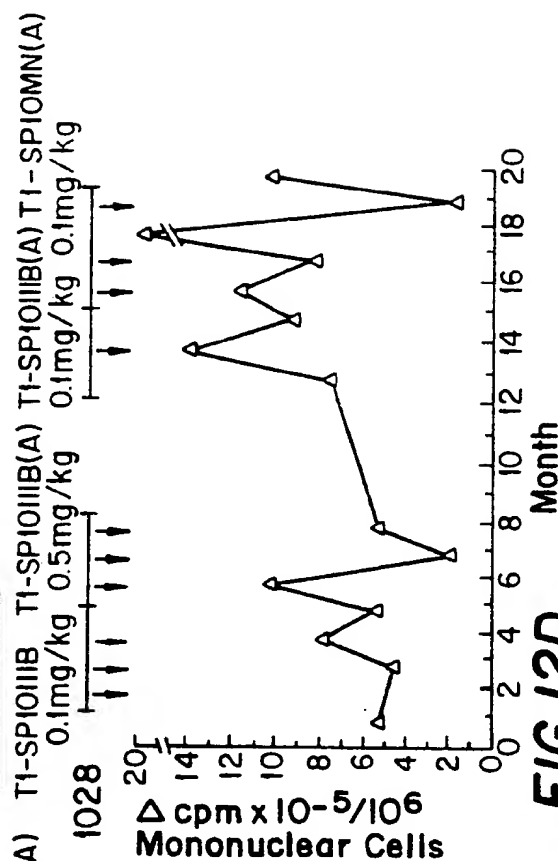


FIG. 12C

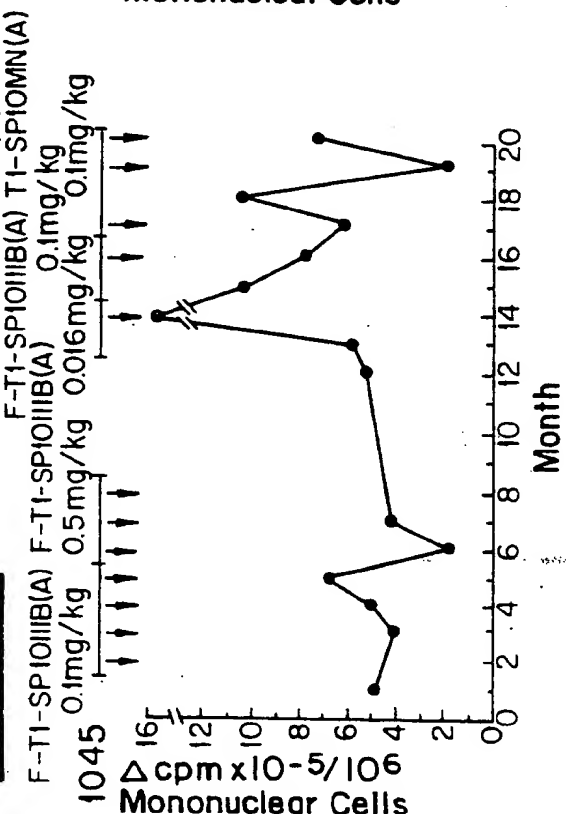


FIG. 12D

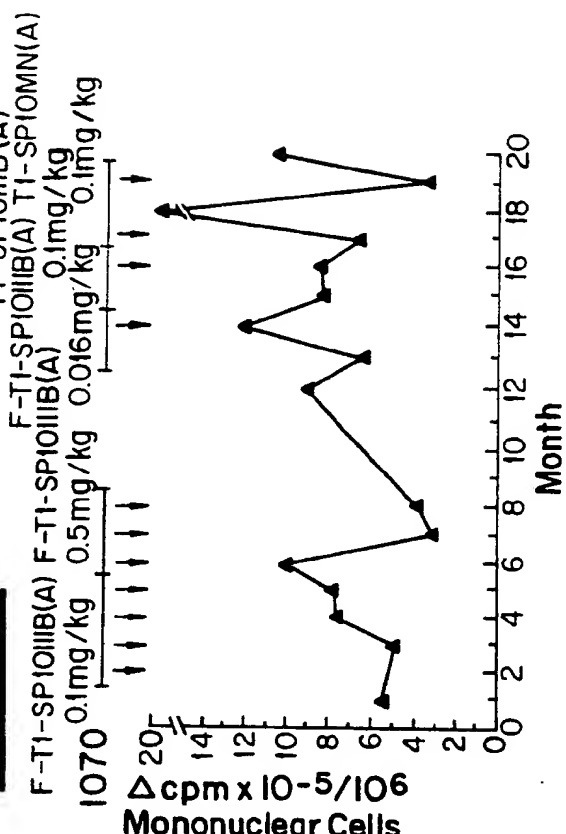


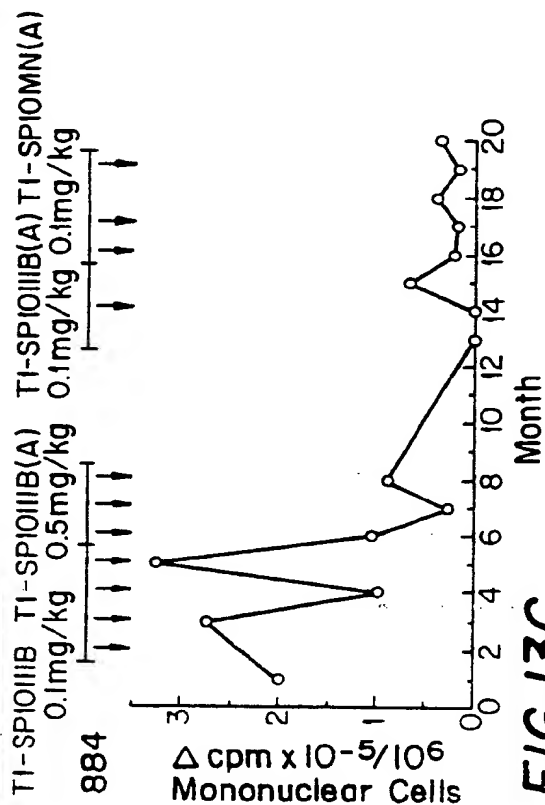
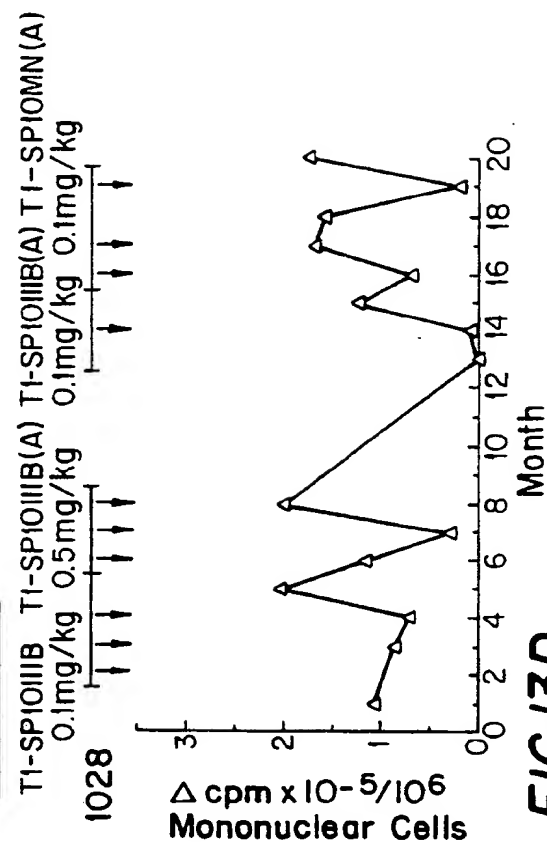
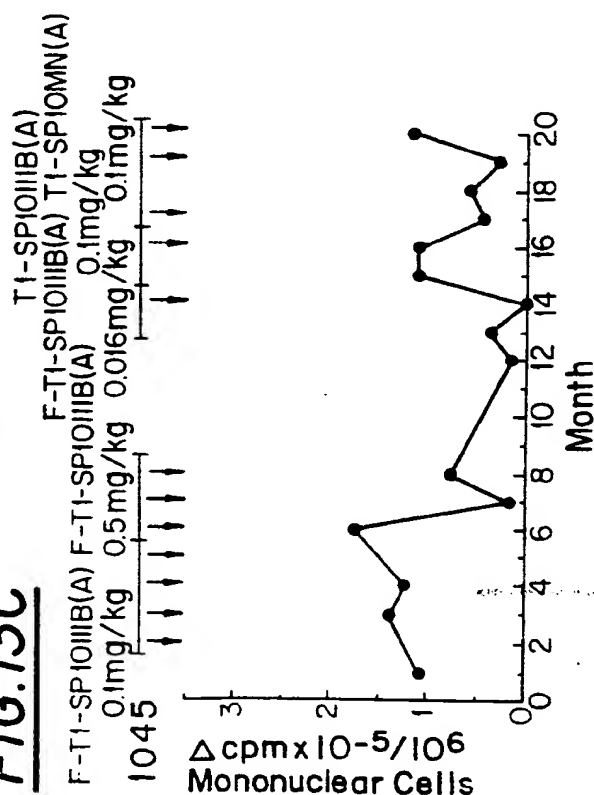
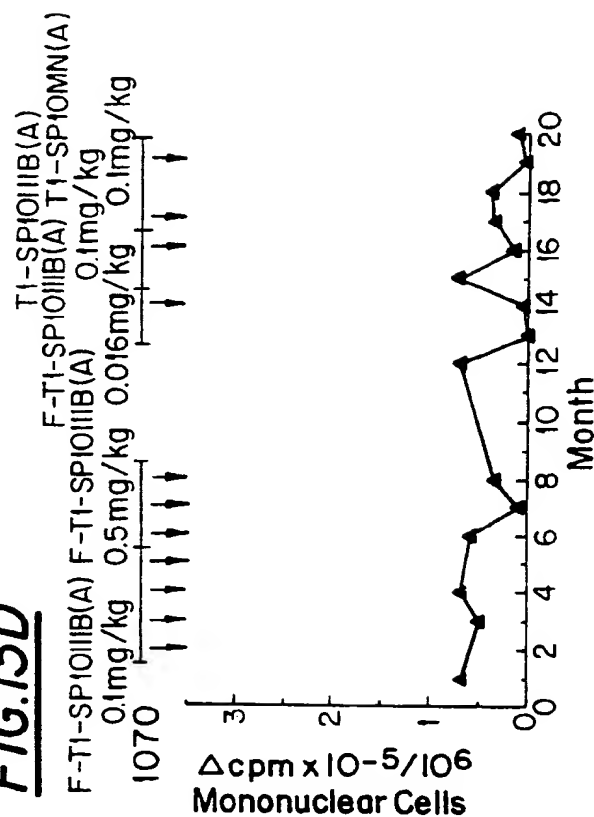
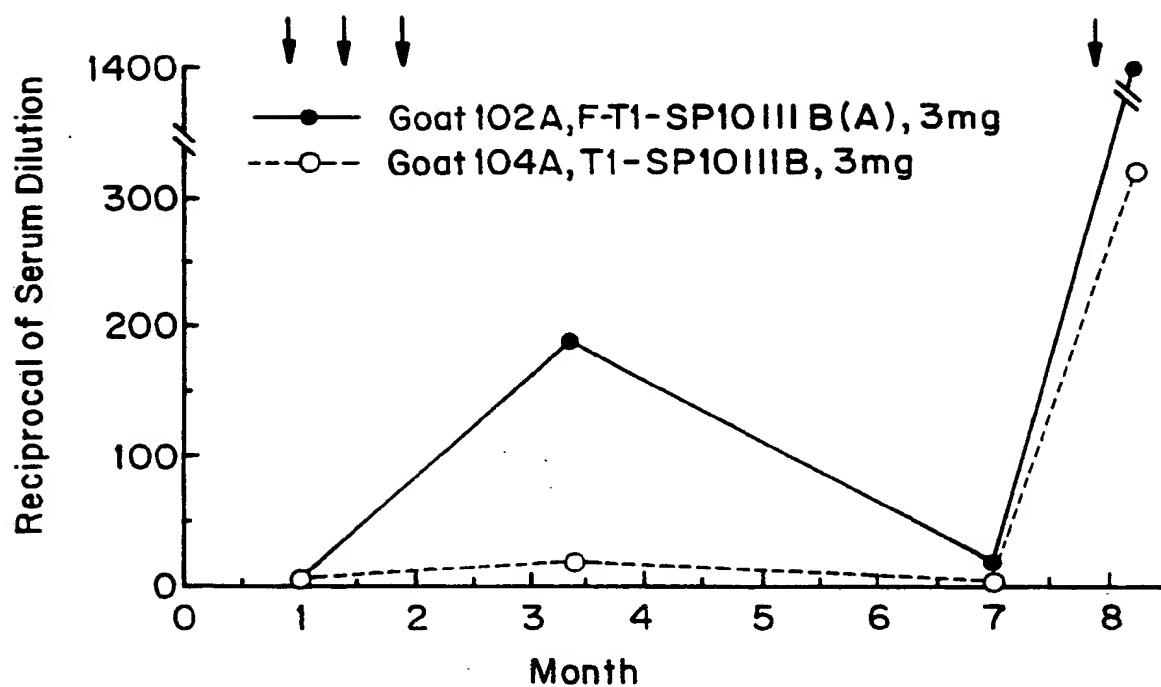
FIG. 13A**FIG. 13B****FIG. 13C****FIG. 13D**

FIG. 15

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/01207

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 37/00, 37/02, 39/12; C07K 5/00, 7/00, 15/00, 17/00

US CL :530/324, 327; 514/12, 14; 424/89

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/324, 327; 514/12, 14; 424/89

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CA, BIOSIS, STN

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, VOL. 88, ISSUED NOVEMBER 1991, HART ET AL, "PRIMING OF ANTI-HUMAN IMMUNODEFICIENCY VIRUS (HIV) CD8+ CYTOTOXIC T CELLS <u>IN VIVO</u> BY CARRIER-FREE HIV SYNTHETIC PEPTIDES", PAGES 9448-9452, SEE ENTIRE DOCUMENT.	1-15
Y	BIOCHEMISTRY JOURNAL, VOL. 269, ISSUED 1990, PAPADOULI ET AL., "ANTIGENIC ROLE OF SINGLE RESIDUES WITHIN THE MAIN IMMUNOGENIC REGION OF THE NICOTINIC ACETYLCHOLINE RECEPTOR", PAGES 239-245, SEE ENTIRE DOCUMENT.	1-15

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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A document defining the general state of the art which is not considered to be part of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z*	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 28 April 1993	Date of mailing of the international search report 04 MAY 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer AVIS DAVENPORT
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/01207

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOURNAL OF EXPERIMENTAL MEDICINE, VOL. 167, ISSUED MARCH 1988, GOLDING ET AL., "IDENTIFICATION OF HOMOLOGOUS REGIONS IN HUMAN IMMUNODEFICIENCY VIRUS I gp41 AND HUMAN MNC CLASS II B 1 DOMAIN", PAGES 914-923, SEE ENTIRE DOCUMENT.	1-15
Y	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, VOL. 171, NO. 1, ISSUED 31 AUGUST 1990, MURAKAMI ET AL., "IDENTIFICATION OF IMMUNOGENIC REGIONS IN HUMAN THYROTROPIN RECEPTOR FOR IMMUNOGLOBULIN G OF PATIENTS WITH GRAVES DISEASE", PAGES 512-518, SEE ENTIRE DOCUMENT.	1-15

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